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THE UNIVERSITY OF ALBERTA

THE EFFECTS OF PREGNANCY ON BILE ACID TURNOVER IN THE RAT

by

Gerald C. O'Sullivan

©

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled THE EFFECTS OF PREGNANCY ON BILE ACID TURNOVER IN THE RAT submitted by Gerald C. O'Sullivan in partial fulfillment of the requirements for the degree of Master of Science in Experimental Surgery.

G. F. Bourdar
Supervisor

Date July 4, 1975

DEDICATION

This thesis is dedicated to my wife, Breda,
for her patience, love and support.

"Knowledge is not a loose-leaf notebook of facts. Above all, it is a responsibility for the integrity of what we are, primarily of what we are as ethical creatures. The personal commitment of a man to his skill, the intellectual commitment and the emotional equipment working together as one has made the 'Ascent of man'."

J. Bronowski

ABSTRACT

Epidemiological studies have shown that pregnancy and sex hormones are important determinants of an increased susceptability to gallstones. Cholesterol is the main constituent of 80% of the biliary calculi encountered in Western Countries and their formation is preceded by a disturbance of the physico chemical properties of bile which may result from a reduced size of bile salt pool. Anovulants (estrogen-progestin combinations) have been found to increase the lithogenicity of bile and reduce bile acid synthesis and pool size. Numerous studies of bile composition during pregnancy in humans have yielded conflicting results while in experimental animals no impairment of cholesterol solubility has been found.

The effect of pregnancy on bile acid metabolism was studied in rats by an acute bile fistula washout technique. The experimental groups were as follows: a control group of virgin females and a first pregnancy group of similar age and initial body weight.

It was found that pregnancy caused a significant reduction in bile acid synthesis which resulted in a marked reduction in the size of the bile acid pool. The enterohepatic cycle frequency of the bile acid pool was unchanged and therefore there was a reduction in bile salt secretion rate in pregnancy. Bile phospholipid and cholesterol secretion rates

were also reduced in the pregnant group probably as a consequence of the lowering of bile salt output. In this animal model pregnancy did not adversely influence cholesterol solubility.

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CHAPTER I

INTRODUCTION

Epidemiology and Prevalence of Gallstones

A major health hazard in economically developed countries relates to gallstones and their complications. In 1968, it was estimated (1) that 15 million Americans had cholelithiasis but recent data compiled by Heaton (2) indicates that presently this figure is in excess of 20 million (3). The geographic, historical and social patterns and the increasing incidence (3) of this disease would suggest environmental, particularly either excesses or deficiencies in diet (4), and possibly genetic factors (5) as aetiologic agents.

However, prevalence data accumulated since the early 20th century (2,6,7,8,9) has shown that age and sex are also important determinants of the incidence of gallstones. The disease occurs more than twice as frequently in females as in males under 50 years of age and the incidence of calculi is greater in multiparous than in nulliparous women of child

bearing years (7). Nilsson found in Sweden that the pre-pubertal incidence of gallbladder disease was similar in both sexes whereas at puberty there occurred a sharp rise in female preponderance (10). This sex difference remains until the female menopause and subsequently tends to disappear. Two recent prospective epidemiological studies confirmed these findings and found a correlation between disease incidence and parity (11,12).

These observations indicate that sex hormones and pregnancy may be the cause of, or a least be instrumental in the sex difference. This argument is reinforced by recent evidence demonstrating that women of child bearing age, who use oral contraceptives, are twice as likely to have cholelithiasis as women of similar age who do not use the 'pill' (13). Furthermore, women who take oestrogens for menopausal symptoms have a risk factor 2.5 times greater than women not receiving hormones (14). Experimental biliary calculi can be produced with greater ease in female than in male mice (15) while administration of high doses of oestrogens and progesterone to rabbits lead to the formation of gallstones (16).

Current research indicates that the primary event in the pathogenesis of gallstones is the secretion of an abnormal (lithogenic) hepatic bile as a result of a disturbance in bile acid metabolism (3). As outlined in Chapter II, experimental studies on primates and humans have demonstrated a tendency for female sex hormones to produce or accentuate this abnormality, thus partly explaining the increased incidence of gallstones in females.

The Problem

The reason for the increase in gallstone incidence due to pregnancy remains unexplained. Minimal data on bile lipid composition during pregnancy in humans or laboratory animals has been collected, and the effects of parity on bile acid metabolism have not been studied.

Objectives

The purpose of this study is to try to find answers to the following questions:

1. What is the effect of mammalian pregnancy on bile lipid composition?
2. From our studies is there any evidence that pregnancy alters the lithogenicity of bile?
3. What is the effect of pregnancy on the important indices of the enterohepatic circulation of bile acids such as pool size, synthetic, secretion and absorption rates and the enterohepatic cycle frequency of bile acid pool?
4. In what way does pregnancy effect the secretion rate of bile phospholipids and cholesterol?

CHAPTER II

LITERATURE REVIEW

Cholesterol is the main constituent of 80-85% of the biliary calculi encountered in Western countries (3). This review therefore will attempt to analyse and explain how this material is maintained in solution in bile, how it precipitates out of solution, what metabolic factors govern the bile composition, and how these metabolic factors may be altered by female sex hormones and pregnancy.

Physicochemical State of Bile Lipids in Gallstone Formation

Cholesterol is insoluble in water (17) but is maintained in aqueous solution in bile by molecular association with bile salts and lecithin (17,18). Lecithin is insoluble in water but with the function of a liquid crystalline phase, 1 molecule of lecithin can solubilize 1 molecule of cholesterol (19). Bile salts are natural detergents and thus disperse the lecithin cholesterol liquid crystals into small aggregates called mixed micelles (19,20) enabling cholesterol to be excreted harmlessly into the intestine via the biliary

tract. In a bile salt cholesterol mixture one hundred moles of bile salt can solubilize 3 moles of cholesterol but when lecithin is present the solvent capacity of the mixture increases threefold (3). The mixed micelles of bile acid, phospholipid and cholesterol have a limited capacity for cholesterol solubilization which is determined by the relative proportion of each lipid present. Maximum cholesterol solubilization occurs in model solutions with a ratio of two to three molecules of bile salt to one of lecithin (3). Admirand and Small introduced a technique for demonstrating the interrelationship of the three biliary lipid components by plotting the molar concentration ratios of bile salts, phospholipids and cholesterol on triangular co-ordinates (20) (Fig. 1). The three component diagram was constructed from in vitro studies of bile lipid mixtures in water, and the line A.B.C. on the graph (Fig. 1) defines maximum cholesterol solubility obtained from model ternary solutions of bile salts, cholesterol and lecithin in water with a total solid content greater than three per cent. At all points below the line A.B.C. cholesterol is in a true solution or micellar state, while above the line bile is supersaturated with cholesterol and crystalization occurs. Normal human bile is within the micellar zone whereas bile of patients with gallstones is usually supersaturated with cholesterol and falls outside the micellar area (20,21,22). Crystallization of cholesterol in bile is thought to be the primary stage of gallstone formation, and is usually found microscopically in supersaturated hepatic

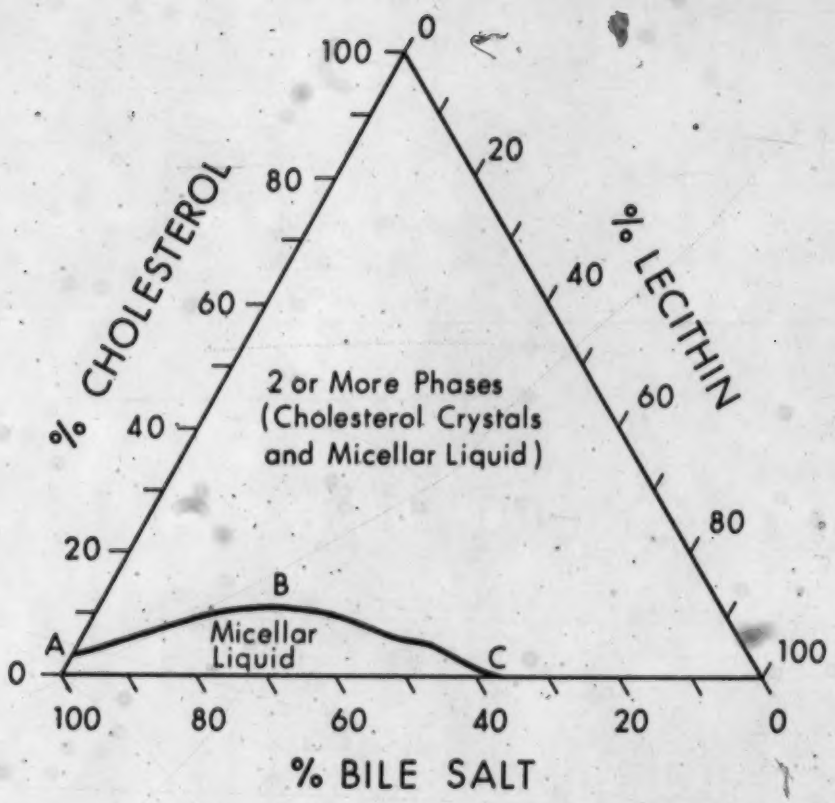


FIGURE 1

TRIANGULAR PHASE DIAGRAM OF BILIARY LIPID COMPOSITION

MAXIMUM CHOLESTEROL SOLUBILITY LIMIT IS DEFINED BY LINE A.B.C.

From Admirand and Small (Ref. No. 20)

bile (after standing) or in saturated gallbladder bile (3,18, 19,20). Consequently an increase in cholesterol or reduction in bile salts or lecithin concentrations could theoretically lead to lithogenic bile formation.

Studies of gallbladder bile in pregnant women at term demonstrated a high incidence of cholesterol crystals with an increase in cholesterol concentration (23,24,25,26). Although the chemical methods used by today's standards are considered inadequate, the finding of crystals is indisputable evidence of cholesterol insolubility. A further study by Large (27) on bile composition in pregnant women failed to confirm the previous reports but when his data was plotted on the phase diagram by Small (17) bile composition fell on the border between the micellar and crystalline regions. Glenn and McSherry, 1969, reported a similar bile composition in pregnant and non pregnant patients all suffering from the same type of gallstones (28). However, that study failed to prove or disprove an influence of pregnancy on the physic-chemical state of bile. as all patients suffering from cholesterol type gallstones would be expected to have similar bile composition.

The baboon spontaneously forms cholesterol gallstones (29) and most normal animal studies have shown a hepatic bile supersaturated with cholesterol; however, the gallbladder bile from the same animals was within the micellar zone, but in pregnancy both hepatic and gallbladder bile were well outside this region (30). While this was a clear demonstration

of the lithogenic influence of pregnancy on gallbladder bile, the finding of normal gallbladder bile in the presence of a supersaturated hepatic bile in control animals requires further investigation. Studies by Martin (31) on the bile composition in pregnant monkeys failed to demonstrate any changes, in contrast to the finding that Estriol increased the lithogenicity of bile when administered to the same animals (32,33). In pregnant rabbits Johnson and Kalant (34) found a decrease in the relative concentration of unconjugated mono and dihydroxy bile acids, and a corresponding increase in conjugated acids in gallbladder as compared to hepatic bile, and concluded that this may be due to the increased time for their absorption as a result of gallbladder stasis. In the guinea pig cholesterol solubility is unaffected by pregnancy, but biliary bile acid concentration is increased at term (35).

Bile Lipid Secretion

Since lithogenicity is a function of the relative concentrations of bile acids, phospholipids and cholesterol in bile the following factors individually, or in combination, could lead to the formation of lithogenic bile:

(a) Decreased secretion of bile acids,

(b) Decreased secretion of phospholipids,

(c) Increased secretion of cholesterol.

The secretion rate of bile acids may be of paramount importance because it may possibly govern the secretion rate of cholesterol and phospholipids by the liver. Biliary

phospholipid and cholesterol secretion rates fall dramatically following acute depletion of bile acids from biliary fistulae in rats (36), and also from the isolated canine liver in the absence of bile salts (37,38). Furthermore administration of bile acids increase lipid secretion rates (39,40). These observations have been confirmed in the monkey (41) and humans (42,43,44,45,46). While bile acid and lecithin secretion rates show a significant correlation, changes in cholesterol output are much less affected by changes in bile salt secretion (41) which suggests that a portion of the cholesterol is secreted independently of bile acids. Furthermore, studies on the regulatory mechanism of biliary lipid secretion by bile acids has revealed a stimulation of lecithin synthesis in the microsomes (38,47) and the latter were also found to be the only source of cholesterol and lecithin in liver. Thus it was suggested by Swell (38) that the bile lipid micelle is assembled at a specific site in the liver microsome, coupled to a carrier protein and transported to the biliary canaliculi. Since lithogenic bile is frequently secreted directly from the liver (24,25) it may result from formation of micelles by microsome with an abnormally high proportion of cholesterol. The bile composition would therefore be regulated by the availability of bile acids to liver cell or by the activity of their hepatocyte transfer system.

In economically developed countries the majority of patients suffering from biliary calculi have reduced bile acid and phospholipid secretion rates and increased cholesterol secretion

rates (48,49,50). A change in enterohepatic cycle frequency or a diminution of bile acid pool size would explain the reduction in bile acid and phospholipid secretion rates. The mechanism of increased cholesterol secretion is unexplained but this may be associated with an increase in synthetic rate (50).

Estriol administration to monkeys acutely reduced bile acid and lecithin secretion (32,33) and the magnitude of this reduction was directly related to the dose of drug administered. Because cholesterol secretion rates remained relatively constant the lithogenicity of bile increased. The immediacy of this change would suggest a mechanism involving an alteration in the hepatocyte transport system rather than prehepatic factors. Investigations along this line reveal that the hepatic transfer maximum of bile salts and bile salt secretion is inhibited by ethinyl estradiol administration to rats, (51) but whether reduced secretion by the hepatocyte or enhanced bile salt reabsorption by the intraphepatic biliary tree is the operative mechanism requires clarification. No information is available on the effects of pregnancy on the secretion of bile lipids.

Changes in Bile Acid Pool

The pool of bile acids is defined as the "total mass of bile acids in the Enterohepatic circulation" and both bile acid synthesis and reabsorption from the intestinal lumen have been shown to influence the size of the pool (52). A reduced bile acid pool is frequently found in association with gall-

stones (53,54,55,56,57) and this metabolic defect is considered responsible for the associated cholesterol insolubility, (48,58,59) since re-expansion of the pool by feeding chenodeoxycholic acid improves cholesterol solubility (46,60) in bile and promotes gallstone dissolution (61,62). Bile acid kinetic studies have shown that patients with gallstones have increased turnover of cholic and chenodeoxycholic acids, while the total bile acid synthetic rate is similar to normal values (53,54,55, 56) which suggests that increased bile salt loss is responsible for the diminution of pool size. However, patients with gallstones have been found to have lower activity of the enzyme cholesterol 7αhydroxylase (the rate limiting enzyme in synthesis of bile acids from cholesterol) in their livers, which suggests that impairment of bile acid synthesis may also be contributing to the diminution of pool size (63). In patients with a reduced bile acid pool, bile acid and lecithin secretion rates are lowered while cholesterol secretion rates remain constant (48, 49,50). These changes in bile lipid secretion rates reduce concentrations of bile acids and lecithin in bile while the concentration of biliary cholesterol is unchanged and therefore an increase in lithogenicity occurs. Analysis of data on humans by Swell has revealed that there is a direct correlation between the degree of cholesterol saturation of bile and bile acid pool size (3) (Fig. 2).

Bile acid kinetics and pool size have not been measured in pregnancy. However, anovulatory drugs (estrogens and progestins) were found to increase the saturation of fasting

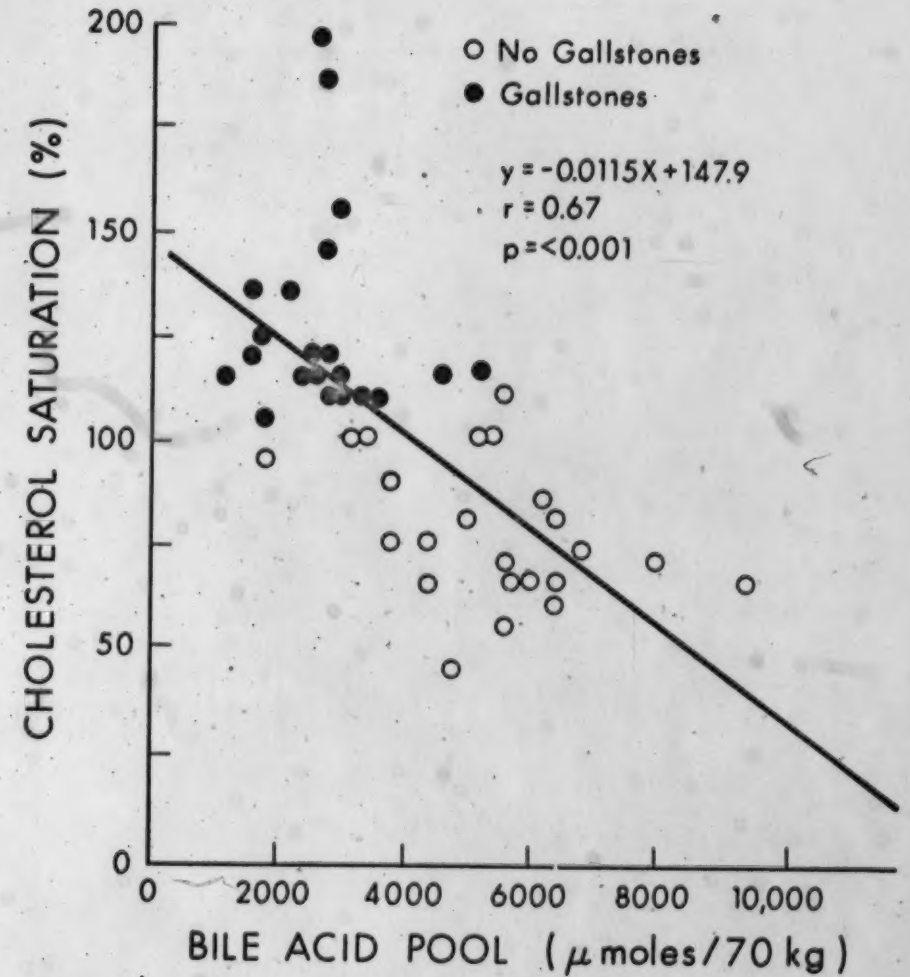


FIGURE 2

CORRELATION OF THE CHOLESTEROL SATURATION INDEX WITH BILE SALT POOL SIZE IN PATIENTS WITH AND WITHOUT GALLSTONES

From Swell et al. (Ref. No. 3)

hepatic bile with cholesterol and to reduce cholic acid pool size and synthetic rate when measured in humans with a choledochostomy (64). Caution must be exercised prior to extrapolating from this study to the gravid state. Hyperplasia of gut with increased absorption potential for carbohydrate occurs during pregnancy but whether or not there is a change in bile acid reabsorption rate is unknown. However, it is possible that increased reabsorption would occur and compensate for any reduction in hepatic synthesis of bile acids and hence prevent a diminution of pool size.

The Enterohepatic Circulation of the Bile Acid Pool

The enterohepatic circulation (EHC) is a physiological entity which serves to provide an endless flow of material from the liver by the biliary tract to intestinal lumen and then back to the liver again. Anatomically the EHC is channeled into two main pathways, the portal EHC and the extra portal EHC (65) (Fig. 3).

In the portal EHC substances secreted by the liver return via portal vein, to be extracted and resecreted by the liver again. Therefore very little of the substance at any time appears in the peripheral bloodstream. Bile acids are thought to undergo primarily a portal EHC. In the extra portal EHC substances secreted by liver are absorbed from intestinal lumen and pass via lacteals into lymphatics and drain into superior vena cava. Cholesterol (66) and to a lesser extent phospholipids (67) undergo an extra portal EHC.

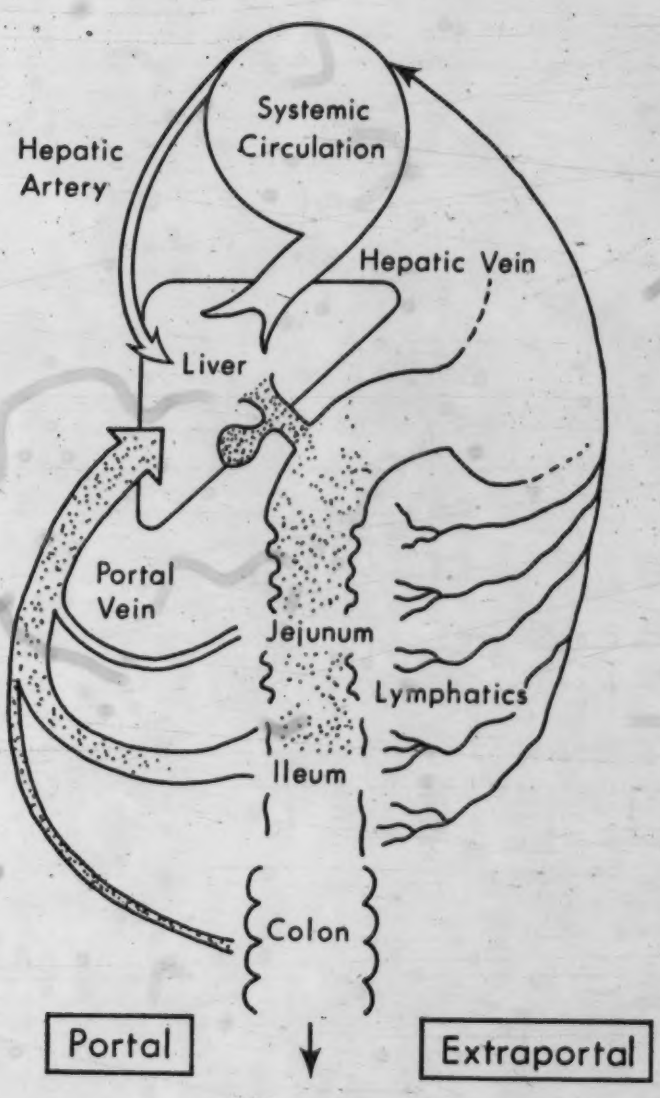


FIGURE 3

THE ANATOMICAL DIVISIONS OF THE ENTEROHEPATIC CIRCULATION

From Small et al. (Ref. No. 65)

The EHC of bile salts is physiologically important in the following ways:

(a) Adequate quantities of bile salts are necessary in gut lumen for digestion and absorption of fat, fat soluble vitamins and cholesterol.

(b) A normal EHC is necessary for regulation of bile salt synthesis from cholesterol in liver (68). Furthermore, by regulating cholesterol reabsorption, bile acids indirectly influence cholesterol synthesis in the intestinal wall and liver as cholesterol controls its own biosynthesis by negative feedback inhibition (69,70).

(c) A normal EHC of bile salts is required for normal hepatic synthesis and secretion of phospholipids.

(d) Normal intact EHC of bile salts is required for normal secretion of free cholesterol from liver into intestinal tract.

Alteration of EHC by interruption, slowing or obstruction are known to change bile composition (Fig. 4).

Interruption of EHC by experiments which lead to loss of bile acid pool increase the lithogenicity of bile of monkeys and humans (41,42,45). Furthermore, resection, bypass or disease of ileum leads to chronic loss of bile acid and the incidence of gallstones increases (70,72). This raises the question whether there are any physiological conditions that may functionally interrupt the EHC. Delay in postprandial gall bladder evacuation occurs during the progesterone phase of the menstrual cycle (42) and from the 4th month of preg-

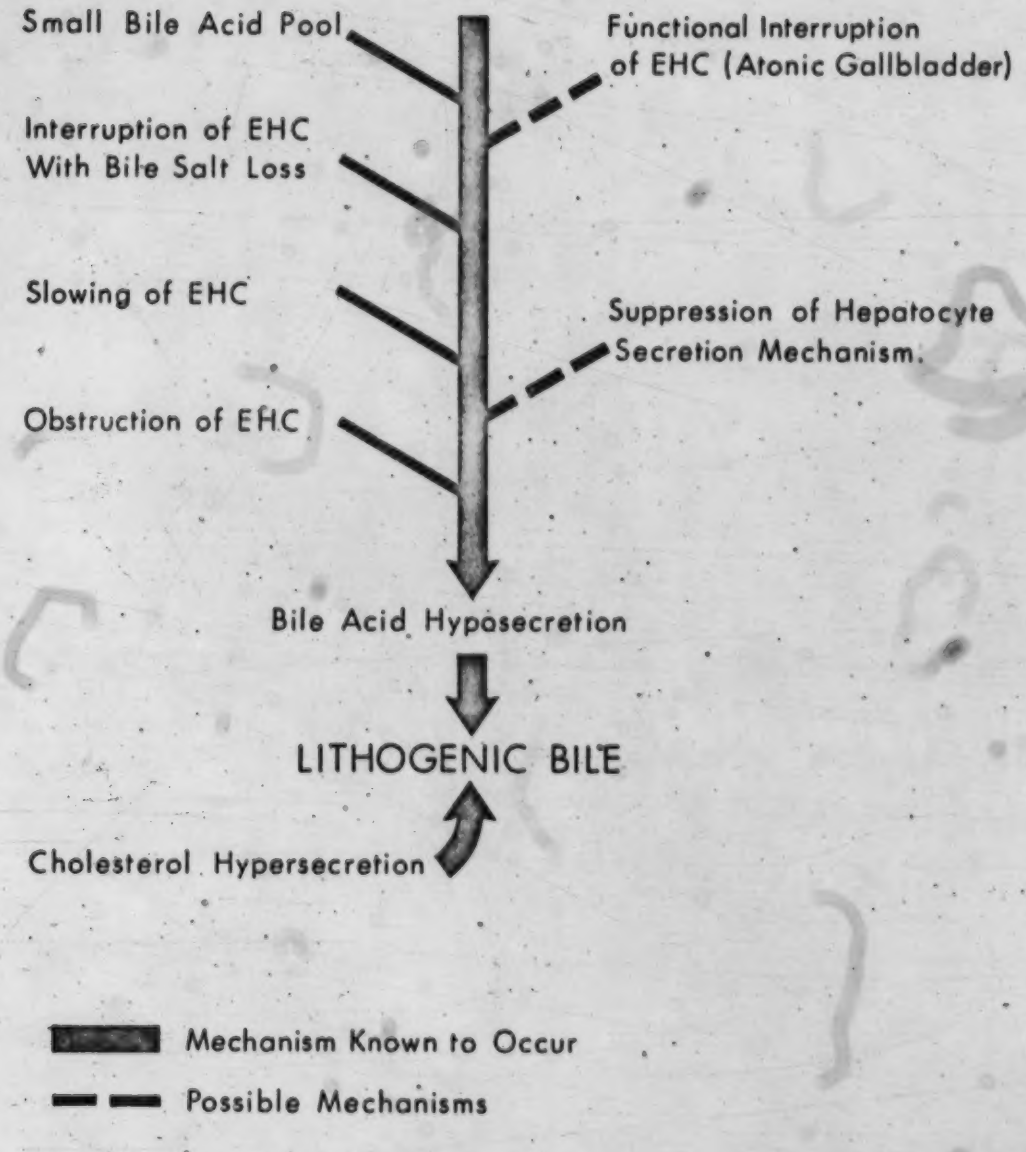


FIGURE 4

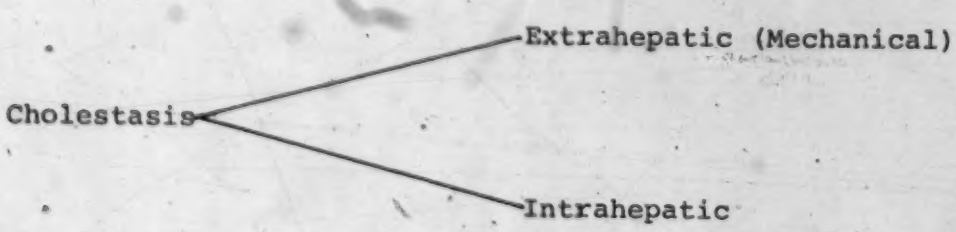
MECHANISM OF SECRETION OF LITHOGENIC BILE BY LIVER

nancy until term (73). This gallbladder atony by sequestering a large quantity of the bile acid pool from the EHC could lead to cholesterol insolubility and gallstone formation. That a functional interruption of EHC may occur is supported by investigations on baboons (74) and humans (49,75) which demonstrate increased outputs of lithogenic bile at night or following prolonged fasting when the gallbladder is in the non contracted state.

Vagotomy and pyloroplasty in monkeys was found to reduce intestinal motility and to slow EHC frequency, however the bile acid pool size remained normal (76). Consequently, a reduction in bile acid secretion rate and increase of lithogenicity of bile resulted (76). Increase of intestinal transit time is well known in pregnancy, but whether this has significant effects on the EHC of bile acids has not been determined.

Cholestasis

Obstruction of enterohepatic circulation (EHC) occurs in high intestinal obstruction, portal vein thrombosis and in biliary stasis (65). As cholestasis is considered relevant to pathogenesis of cholelithiasis and may complicate pregnancy--this condition will be further discussed:



Cholestasis is defined as the syndrome associated with failure of bile to reach the duodenum (77) and is aetiologically separated into two types i.e., extrahepatic and intrahepatic. Common to both forms there is retention of bile acids resulting in abnormal distribution of pool. Furthermore, high concentration of bile acids occur within liver (78,79) and these have been found to suppress bile acid synthesis (65,80).

Mechanical obstruction of the biliary tract may be of importance in lithogenesis as many cases of gallstones are found in association with stenosis of the Ampulla of Vater (81) or may be produced experimentally by chronic biliary stasis in rabbits, monkeys (81) and dogs (81,82). Repeated obstruction of biliary tract of a primate results in lithogenic bile on relief (65,80) which suggests that in situations of intermittent obstruction calculi may result. Westphal (83) is reported to have observed an increase in tone of the sphincter of Oddi during pregnancy but this has not been corroborated.

Because the hepatic structure in pregnancy is normal (80,85,86) the reduced elimination of Bromosulphthalein (87) and rise in serum alkaline phosphatase (88) suggest that suppression of the hepatocyte secretory mechanism occurs. This hypothesis is supported by the observations that estrogen therapy to rats (51,85) and monkeys (32,33) reduces bile salt secretion and bile salt dependent and independent flow rates. Pruritis with elevation of serum bile acids and jaundice may complicate pregnancy or anovulant therapy (77,88), and in these cases the histological (84,85) and ultrastructural

findings (86) are those of cholestasis but the major duct system is normal. The importance of these events in the genesis of gallstones is unclear as bile lipid secretion or bile formation mechanisms have not been studied in the gravid animal.

The Role of the Gallbladder

Gallstones form in the gallbladder (yet the liver is the site of origin of abnormal bile), thus raising the question whether abnormal gallbladder function in any way contributes to the mechanism of calculus production. Theoretically altered gallbladder function might influence gallstone formation by effects on:

(a) Bile acid metabolism and hepatic bile composition.

(b) Bile composition in gallbladder.

(c) Stone growth.

Recent reports of the normalization of bile composition after cholecystectomy suggested that abnormal gallbladder function contributed to the secretion of lithogenic bile by the liver (90,91). Correlation of bile acid pool size with gallbladder capacity and the finding of a reduction in pool size following cholecystectomy suggested that abnormal gallbladder function results in an increased EHC frequency with consequent increased bile salt loss and reduction of bile acid pool size (92). However, it has now been conclusively established that bile remains lithogenic after cholecystectomy (32,33,42,93,94) and a recent investigation by Almond et al.,

found no difference in bile acid pool sizes, or bile acid synthetic rates pre and post cholecystectomy (94). These findings indicate that the metabolic defect in bile acid metabolism leading to cholesterol gallstones is not of gallbladder origin.

It is unlikely therefore that pregnancy would alter bile acid pool size or synthetic rate by its influence on the gallbladder. Interruption of the EHC by sequestration of bile acid in an atonic gallbladder has been postulated as a mechism for the production of lithogenic bile during pregnancy.

A further mechanism whereby the gallbladder may influence lithogenicity of bile is by altering its composition through selective absorption of certain constituents. Bile acid or phospholipid reabsorption has been found to occur from normal gallbladder (95,96) and this is accentuated in the inflamed, distended or ischaemic bladder (95). These mechanisms, possibly explain the finding of a reduced cholesterol holding capacity of gallbladder bile relative to hepatic bile in mice on a lithogenic diet (97). During a prolonged period of gallbladder atony, such as occurs in pregnancy, continued reabsorption of bile acids and phospholipids could lead to the production of lithogenic bile. Support for this theory is offered by the finding of lower levels of conjugated mono and dihydroxy bile acids in gallbladder relative to hepatic bile in the pregnant rabbit (34).

Hydrolysis of lecithin in the gallbladder due to an increase in phospholipase A activity in bile, converting

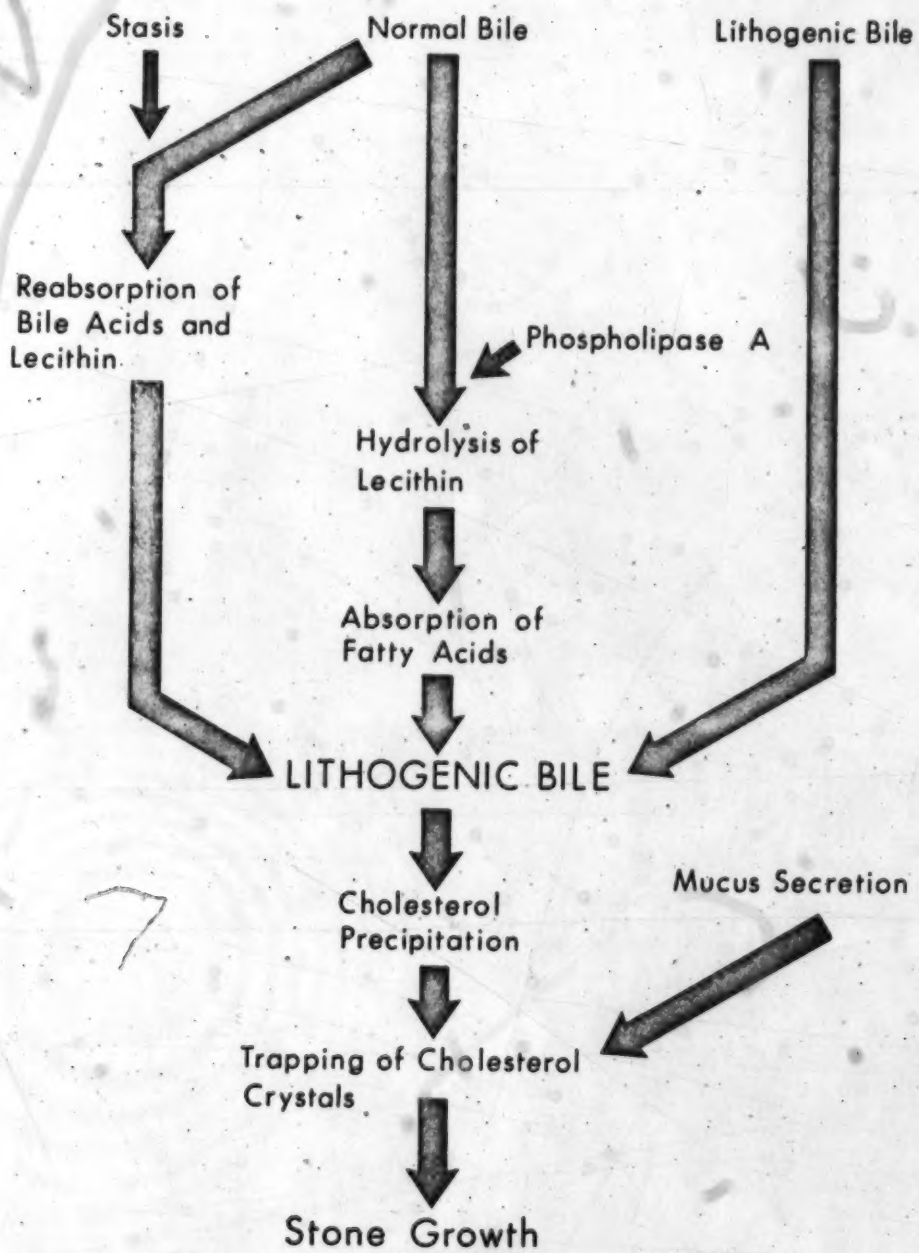


FIGURE 5

EVENTS IN GALLBLADDER THAT MAY LEAD TO THE FORMATION OF LITHOGENIC BILE AND GALLSTONES

significant quantities of lecithin to lysolecithin and fatty acids was proposed by Bouchier (98). Subsequent absorption of the fatty acids by the gallbladder would lead to disruption of micelles and cholesterol precipitation. The finding of Calcium Palmitate in gallstones is considered evidence that lecithin hydrolysis occurs. Phospholipase A is normally present in pancreatic secretions, may also by synthesized by coliform bacteria and has been isolated from gallbladder bile. Supporting the theory of pancreatic reflux into the biliary tract Potter found amylase activity in 31% of gallbladder bile specimens collected from pregnant women (26). The significance, and further proof of its actual occurrence requires more investigation.

A further explanation for the difference in gallstone incidence between parous and nulliparous women could be related to an influence on stone assimulation within the gallbladder. Retardation of emptying may lead to a retention of abnormal bile and subsequent cholesterol crystallization. Also in gallbladder atony stratification of bile into layers with various degrees of cholesterol saturation would lead to its precipitation from the supersaturated strata (99).

An increase in mucus content is found in bile of patients with gallstones (101) and its secretion is increased in gallbladders of rabbits on a lithogenic diet (102). Trapping of cholesterol crystals in the mucosal niches of the gallbladder by the mucus (100) and the subsequent aggregation of the mucus-

cholesterol complexes into clots may be an essential stage in gallstone development (100,101) and this process would be favoured by a flaccid gallbladder. Little is known about the factors that normally govern mucus secretion by gallbladder. However, a recent study found no evidence of an increased gallbladder mucus secretion during pregnancy in rabbits (103).

Carbohydrate Metabolism

The geographic and social distribution of gallbladder disease correlates directly with increased consumption of refined carbohydrates (2,4) and reduced intake of dietary fibre (2,4). Cholelithiasis is also associated with diabetes (6,7,104). An increased frequency occurs in diabetics (104) and also impaired carbohydrate tolerance is a common finding in patients with gallbladder disease (105). Furthermore, experimentally cholelithiasis has been induced in dogs by feeding a sugar rich diet (106). Impaired carbohydrate tolerance occurs also in pregnancy but whether this is causally related to the increased incidence of gallstones is unknown.

Summary

An increased incidence of the cholesterol type gallstone is associated with pregnancy or sex hormone therapy. Abnormal bile composition (lithogenic bile) is a pre-requisite for lithogenesis and this may result from either a reduced bile acid pool size, slowing of enterohepetic circulation of pool, interruption or obstruction of EHC or increased secretion of cholesterol by the liver. A further mechanism by which

lithogenic bile is thought to form is by selective reabsorption of bile acids and phospholipids from gallbladder, associated with hydrolysis of lecithin by Phospholipase A.

Female sex hormones have been found to increase lithogenicity of bile and reduce secretion rate of bile acids in the primate. In humans they result in a reduction of bile acid synthetic rate and pool size with subsequent impairment of cholesterol solubility. Studying the effects of pregnancy on bile composition in humans has yielded conflicting results. Experimentally, lithogenic bile is found in the gallbladder of pregnant baboons but no impairment of cholesterol solubility was found in the gallbladder bile of Rhesus monkeys, rabbits or guinea pigs. The effects of pregnancy on bile acid metabolism or bile lipid secretion are unknown.

From results of studies with anovulants and estrogens it is postulated that the increased sex hormone production in pregnancy may suppress bile acid synthesis reduce pool size, reduce bile acid secretion and increase bile lithogenicity. Pregnancy may also cause a reduction in bile acid secretion because of slowing of EHC, due to the increase in intestinal transit time, or of interruption of EHC due to failure of emptying of gallbladder. Furthermore, alteration of gallbladder function may lead to selective reabsorption of bile acid and phospholipids or to hydrolysis of lecithin thus altering bile composition in the direction of lithogenicity. A flaccid gallbladder would also retain abnormal bile and prevent removal of cholesterol crystals or mucus cholesterol

complexes thus aiding stone growth.

Occurrence of these hypotheses individually or in concert would explain the increased incidence of gallstones due to pregnancy.

CHAPTER III

MATERIAL AND METHODS

Choice of Animal Model

Ideally this study would be more rewarding if it were undertaken on the pregnant human, but the necessity for surgical intervention and/or isotope administration forbids such an exercise.

Studies on primates approximate more to the human situation but an adequate supply of pregnant animals is not available. From the Rodent species the rat was chosen as an animal model because:

(a) The absence of a gallbladder in this species meant that any changes seen in bile acid turnover would be due to the direct influence of pregnancy on the metabolic pathway.

(b) Constant availability of an adequate supply of pregnant animals.

(c) It was possible to plan and accurately diagnose pregnancy, thus ensuring rigid experimental control and facilitating experimental programme planning.

(d) Accurate matching was possible before induction of pregnancy, thus ensuring that any observed changes should be due to the effects of parity.

(e) The hardiness and durability of rat guaranteed a low mortality during the study period.

(f) The literature contains much information on bile acid metabolism and methodology for the rat.

Animals

Female Wistar rats 2 1/2 - 3 months of age and with an initial body weight of 200-250 grams were chosen for experiment.

All rats were purchased from Woodlyn Laboratories Limited, Guelph, Ontario. Each month a group of animals were taken and 50% were randomly chosen for induction of pregnancy. Pregnancy was confirmed by the vaginal plug method. Gravid animals at 12 days gestation and matched controls were transported simultaneously but in separate containers to the University of Alberta.

At the Surgical Medical Research Institute, the animals were housed in plastic cages in groups of four and were allowed water ad libitum and were fed Purina rat Chow. The room temperature was constant at 19-21°C and relative humidity at 35-40%. Lighting was controlled by a time switch, maintaining 12 hours daylight between 6:30 and 18:30 hours.

Before experiment the animals were allowed 7 days to adapt to the controlled environmental conditions.

Bile Acid Metabolism Studies

The bile acid pool size, secretion and hepatic basal synthetic rates were measured in an acute bile fistula preparation, similar to Mok et al., (1974)(52) using Myant and Eder's (1961)(107) modifications of Eriksson's (1957)(108) washout technique. The validity of assumptions made on this experimental preparation have previously been verified experimentally in bile fistula Rhesus monkeys (41,109).

Experimental Groups

There were two groups of rats in this study:

(a) A control group of virgin females maintained under similar environmental conditions to pregnant group and studied simultaneously.

(b) A first pregnancy group at 19 days gestation.
The 19th day of pregnancy was considered the most suitable time for study in the rat because it should represent more closely the hyperoestrogen phase in last trimester of human pregnancy than any earlier phase and studies carried out later would be complicated by parturition. Also it was easier to adjust experimental protocol to this time.

Surgery

To avoid the effects of starvation on bile acid metabolism, all the animals were studied in the non fasting state and to exclude changes in diurnal rates of bile acid synthesis, each experiment was begun at a constant time.

Anaesthesia was achieved by ether inhalation. A laparotomy was done via an upper midline incision 3cms. in length. Pregnancy was confirmed where relevant, by inspection of uterine horns. The distal end of the common bile duct was isolated, ligated and the proximal end was cannulated with a fine polyethylene cannula (P.E.10 .024" O.D. Portex polyethylene tubing) 18" in length and secured there by circumferential ligaturing of bile duct. The distal end of cannula was then inserted into the duodenum and secured there in order to maintain continuity of the enterohepatic circulation until collection commenced. The loop of tubing was exteriorised via a subcutaneous tunnel at the basi occiput of the rat. The abdomen was closed with continuous 4-0 silk to linea alba and skin. The rat was then placed in a Bollman restraining cage (110) wherein it rapidly recovered from anaesthesia. During experiment the animal was allowed 4.3% Dextrose in 0.3% saline and food pellets ad libitum.

Bile Collection

The one hour observation period was adapted for the experiment as in an earlier pilot study it proved the most convenient and results were similar to those when collection was commenced immediately or after 3 hours.

Bile was collected at two hourly intervals for a total of 22-24 hours by means of an automatic fraction collector. As the first specimen was for determination of bile composition this was collected in a test tube maintained at 0°C by sur-

rounding ice packs and immediately frozen prior to transport to the laboratory in order to inhibit any lecithinase activity known to be present in bile at room temperature.

All animals were weighed before and after experiment. At conclusion of experiment the animals were sacrificed. The livers and uteri were excised and their wet weights determined.

Biochemical Analysis

The volume of each specimen was determined prior to freezing. Cholesterol phospholipid and bile acid levels were determined in the first two hour sample and total bile acid levels on each subsequent two hour collection. The cholesterol and phospholipid levels were determined within 4 hours of collection. The remainder of specimens were stored at -20°C until bile acid analysis.

Bile Acid Concentrations were determined enzymatically using Engert's and Turner's (111) modification of Turnberg's and Anthony-Moate's 3 α hydroxysteroid dehydrogenase technique for total bile acids (112).

Bile Phospholipid was calculated from the total lipid phosphorus content of bile (113) after chloroform methanol lipid extraction (114).

Bile Cholesterol was determined by the Abell (115) method for total cholesterol in serum as recommended by Tonks (116).

Bile Acid Secretion Rate

The bile acid secretion is defined as the total bile acid output per 24 hours (mainly due to recycled but also to a lesser extent due to newly synthesized bile acids) as calculated by extrapolation from the output during first two hours after cannulation of bile duct. The validity of this extrapolation has been verified by studies done in Rhesus monkeys using a technique of controlled interruption of the EHC. With that preparation it was shown that the bile acid secretion rate during the first 2 hours after interruption of EHC approximates closely to the normal secretion rate measured over the preceding 5 to 7 days in the steady state with a virtually intact EHC (41,109).

Bile Acid Pool Size

The bile acid pool may be defined as the total mass of bile acids in the EHC (μ moles/100 gms.B.W.). In this preparation it is the amount of bile acid obtained from the time of creating the fistula until the 'low point' on the washout curve, minus the contribution due to basal hepatic synthesis. (Fig. 6).

Bile Acid Synthetic Rate

The initial secretion rate after fistula creation remains until bile acids in the EHC have been washed out (bile acid pool). Once washout has occurred, secretion reaches a low point when any bile acid appearing has been newly synthesized by the liver. This represents the basal hepatic synthesis and

extrapolated to and expressed as a 24 hour value (Fig. 6).

Absorption Rate of Bile Acids

The bile salts secreted by the liver consist of two components newly synthesized and a larger fraction which has been transported there by the liver i.e. a fraction which has ultimately been absorbed.

Assuming a steady state, absorption rate of bile acids may be determined by subtracting the synthesized fraction from the secreted value. This extrapolation is a measure of the quantity of bile acids absorbed and does not reflect the absorption capacity of gut.

Absorption Efficiency of Bile Acids

In order to determine whether the changes in absorption result from alteration of the ileal active transport system or the availability of bile acid, the absorption efficiency was calculated, i.e. the ratio of quantity of bile acid absorbed to the quantity of bile acid that is available for absorption expressed as a percentage.

$$E\ \% = \frac{A}{B}$$

E = Efficiency of absorption
A = Absorption rate
S = Secretion rate

Enterohepatic Cycle Frequency

Since the secretion rate is the product of pool size and the number of times the pool circulates per day, and since both secretion rate and pool size may be determined, the circulation frequency (number of EH cycles per 24 hours) may be

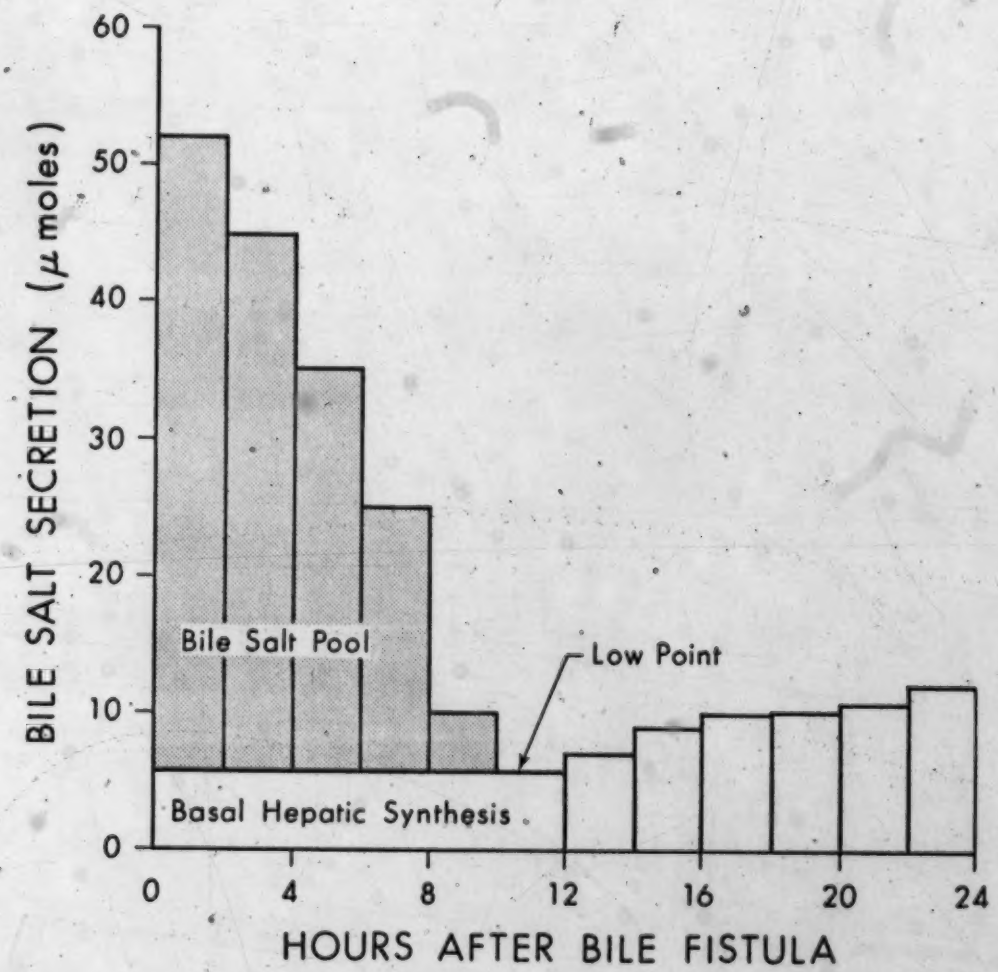


FIGURE 6

BILE SALT SECRETION PATTERN DURING FIRST 24 HOURS AFTER CANNULATION OF BILE DUCT OF RAT

calculated by dividing secretion rate by pool size.

Bile Lipid Composition

This was determined by expressing the results for individual lipids in first 2 hour bile sample as a molar ratio (Moles %) of all three lipids.

Bile Cholesterol and Phospholipid Secretion Rates

These were extrapolated to 24 hour values from the outputs during the first 2 hours of collection.

Bile Volume/24 hours

Daily bile volume was extrapolated from the volume collected over the first 12 hours as recommended by Mok et al.,(52).

Statistical Analysis

The mean values, standard deviation and standard error of mean were calculated routinely for all parameters.

The significance of differences between mean values was analysed with the non paired Students 't' test. A difference was considered significant when the probability (p) value was less than 0.05 (5%).

CHAPTER IV

RESULTS

The animals tolerated the procedure well and there was no mortality. Each animal lost on average 25 grams body weight over the 24 hr. collection period, but appeared to eat and drink adequately. Accidents such as dislodgement of cannula or spillage, were few and when they occurred the animal was rejected from study. At autopsy on cross examination there was no evidence of intra abdominal leakage, pancreatitis, stress ulceration or intestinal obstruction.

It was originally intended to have 30 animals in both groups but at conclusion of the experiment 29 control and 24 pregnant animals were available for study.

In all animals the bile salt secretion pattern was similar to Fig. 6 and there was no difficulty with interpretation or calculation of the various parameters. However, in a number of animals an inadequate volume of bile was produced in the first 2 hours to facilitate quantitative determination of the cholesterol and phospholipid levels.

Liver Mass

There was no statistical difference between the fresh weights of livers as determined at conclusion of experiment (Table I).

Bile Secretion

Bile secretion output remained remarkably constant throughout the 24 hour collection period despite the fall in bile salt secretion. Bile volume/100 gms B.W./24 hours was similar in both groups (Table II).

The individual bile lipid secretion rates were each significantly reduced in the pregnant group, bile salt secretion by 22% (Fig. 7 Table III), phospholipid output by 37% (Fig. 8 Table III), and cholesterol by 40% (Fig. 9 Table III).

Bile Salt Pool Size and EHC Frequency

The pool size of bile acids was measured in 28 control and 22 pregnant animals and was found to be significantly reduced by 38% in the gravid group (Fig. 10 Table IV). There was no difference in EHC frequency of bile acid pool between the two groups (Table V) and the normal EHC rate of the low bile acid pool would appear to be the cause of the reduction in bile acid secretion rate.

LIVER MASS Grams/100g B.W.

	CONTROL	PREGNANT
No. of Rats	29	24
Mean	3.74	3.90
Std. Dev.	0.58	0.54
S.E.M.	0.11	0.11
t value	-	1.0082
p value	-	N.S.*

TABLE I

THE EFFECT OF PREGNANCY ON THE FRESH LIVER WEIGHT IN THE RAT

BILE VOLUME SECRETION Mls/100g B.W./24 hrs.

	CONTROL	PREGNANT
No. of Rats	26	22
Mean	6.60	7.10
Std. Dev.	2.00	1.31
S.E.M.	0.39	0.28
t value	-	1.0550
p value	-	N.S.*

TABLE II

THE EFFECT OF PREGNANCY ON BILE SECRETION IN THE RAT

*N.S.: $p > .05$

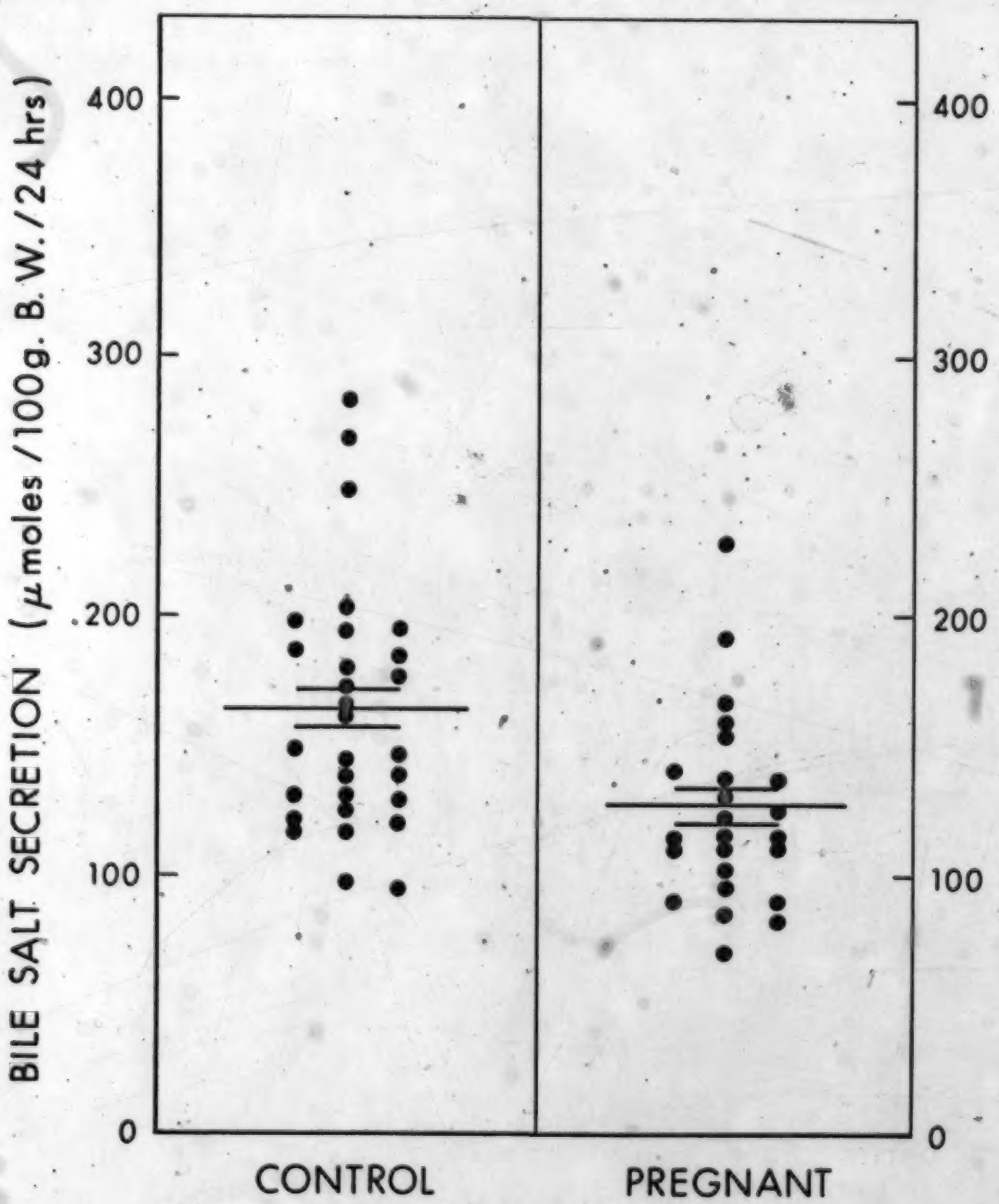


FIGURE 7

BILE SALT SECRETION RATES (MEAN ± S.E.M.)

IN CONTROL AND PREGNANT RATS

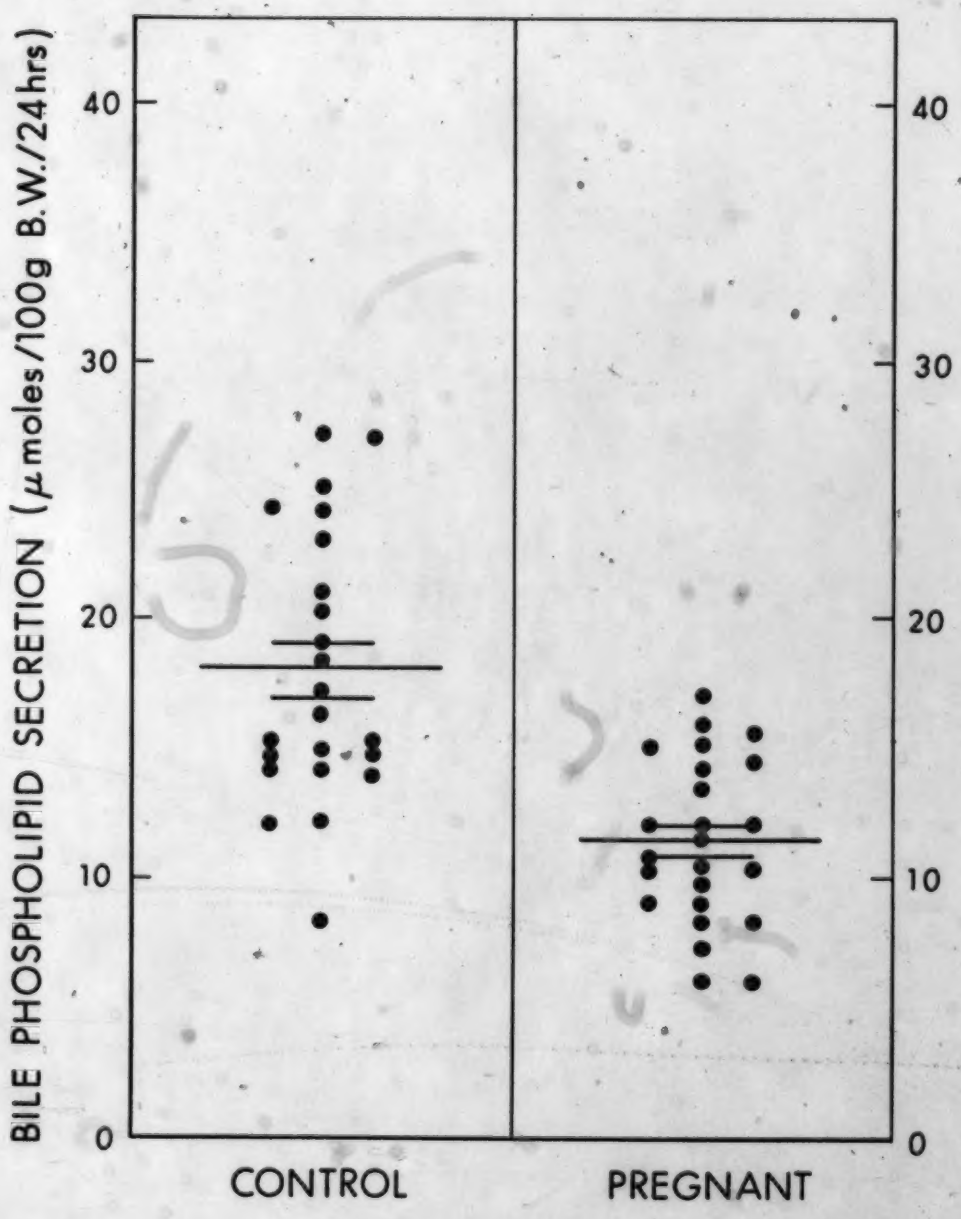


FIGURE 8

BILE PHOSPHOLIPID SECRETION RATES

(MEAN ± S.E.M.) IN CONTROL AND PREGNANT RATS

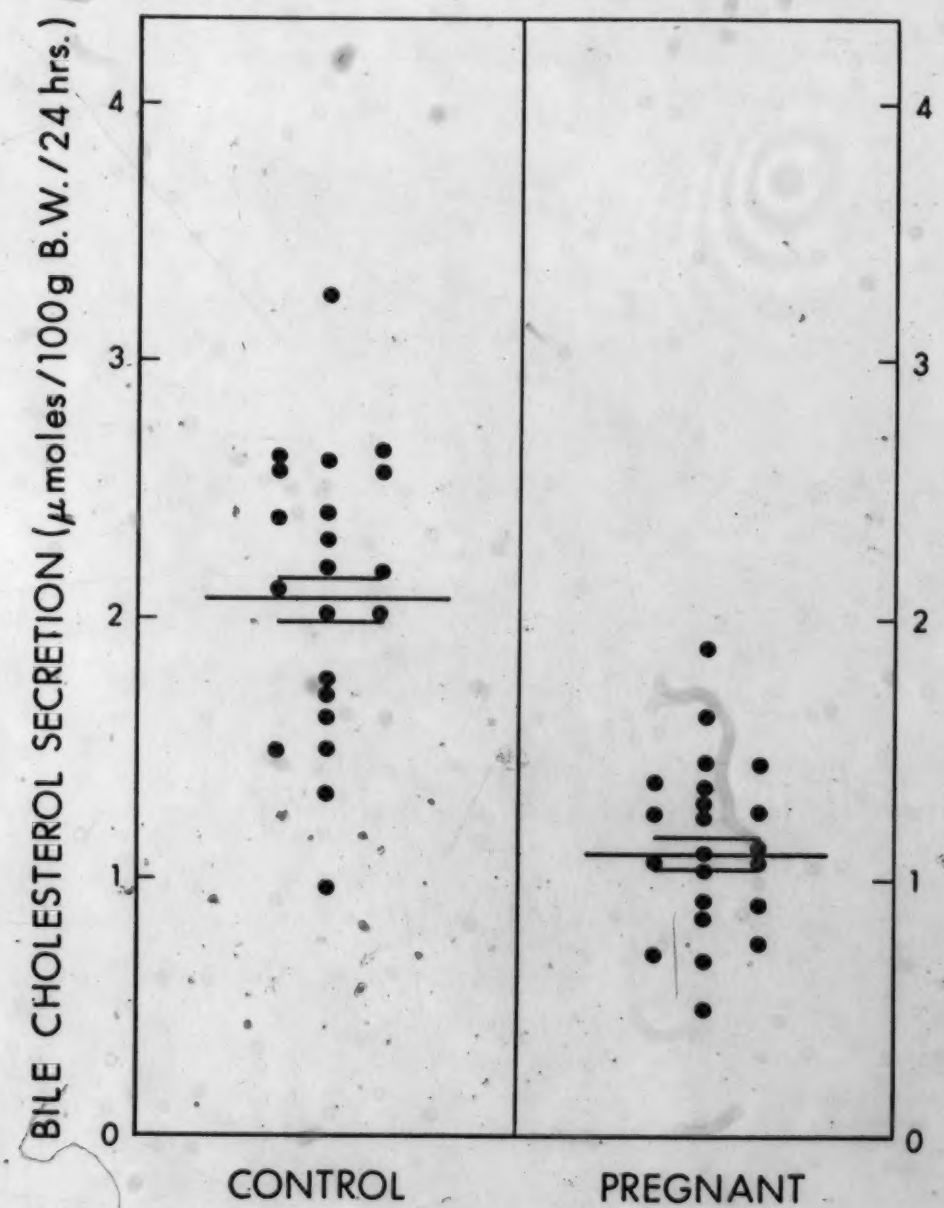


FIGURE 9

BILE CHOLESTEROL SECRETION RATES

(MEAN ± S.E.M.) IN CONTROL AND PREGNANT RATS

BILE LIPID SECRETION RATES (μ MOLES/100g B.W./24 hrs.)

GROUP	BILE SALT	PHOSPHOLIPIDS	CHOLESTEROL
CONTROL			
No.of Rats	29	23	21
Mean	163.39	18.03	2.11
Std.Dev.	47.18	5.24	0.56
S.E.M.	8.76	1.09	0.12
PREGNANT			
No.of Rats	24	24	22
Mean	127.92	11.54	1.14
Std.Dev.	35.49	3.20	0.34
S.E.M.	7.24	0.65	0.07
t value	2.9854	5.0194	6.7454
p value	<.01	<.001	<.001

TABLE III

THE EFFECT OF PREGNANCY ON BILE LIPID SECRETION

IN THE RAT

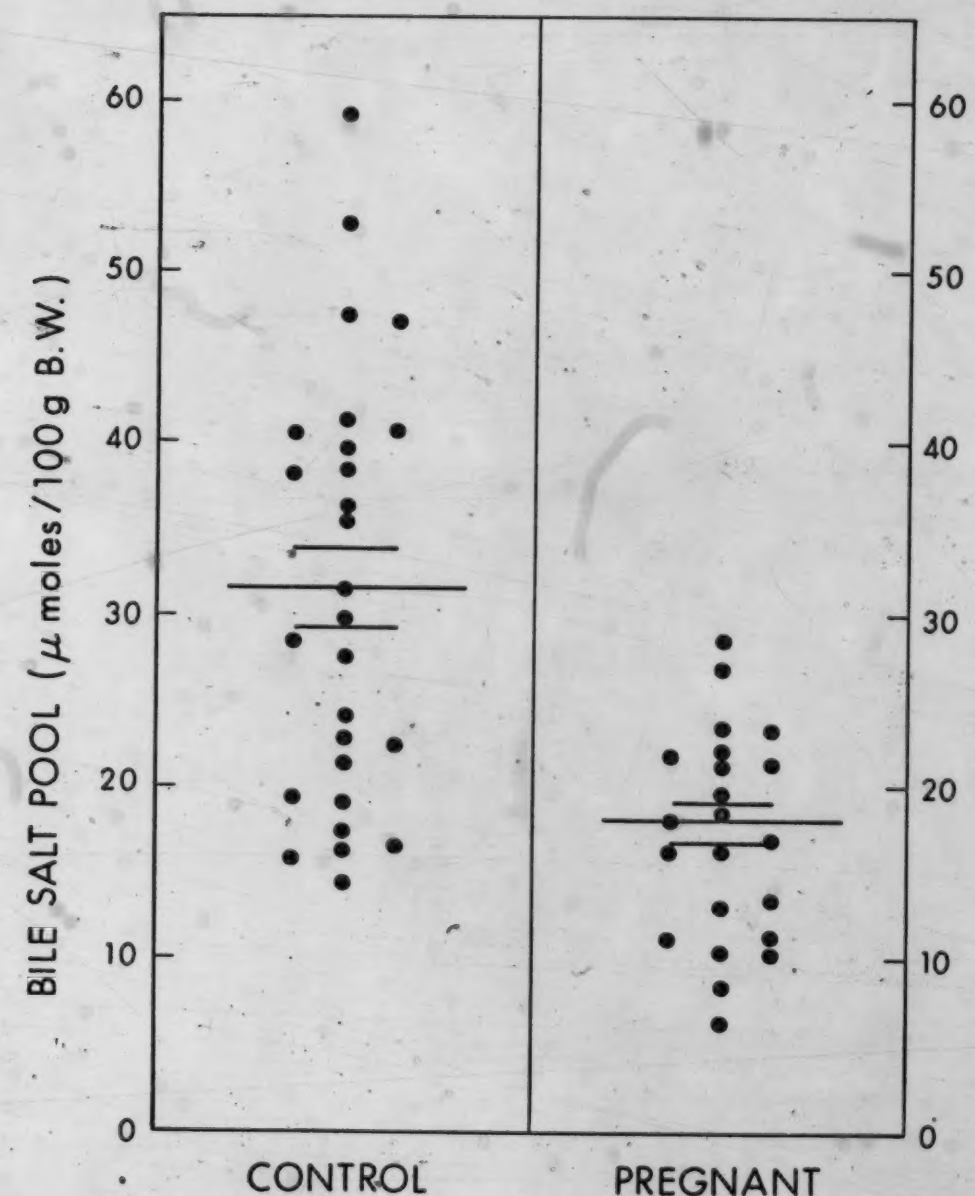


FIGURE 10

BILE SALT POOL SIZE

(MEAN ± S.E.M.) IN CONTROL AND PREGNANT RATS

BILE SALT POOL µMOLES/100g B.W.

	CONTROL	PREGNANT
No. of Rats	28	22
Mean	31.47	18.19
Std. Dev.	12.20	5.19
S.E.M.	2.30	1.11
t value	-	4.6841
p value	-	<.001

TABLE IV

THE EFFECT OF PREGNANCY ON THE TOTAL BILE SALT POOL IN THE RAT

ENTEROHEPATIC CYCLE FREQUENCY OF BILE SALT POOL/24 hrs.

	CONTROL	PREGNANT
No. of Rats	27	22
Mean	6.0	6.8
Std. Dev.	2.12	1.71
S.E.M.	0.41	0.36
t value	-	1.4576
p value	-	N.S.*

TABLE V

THE EFFECT OF PREGNANCY ON THE EHC FREQUENCY OF BILE SALT POOL IN THE RAT

*N.S.: $p > .05$

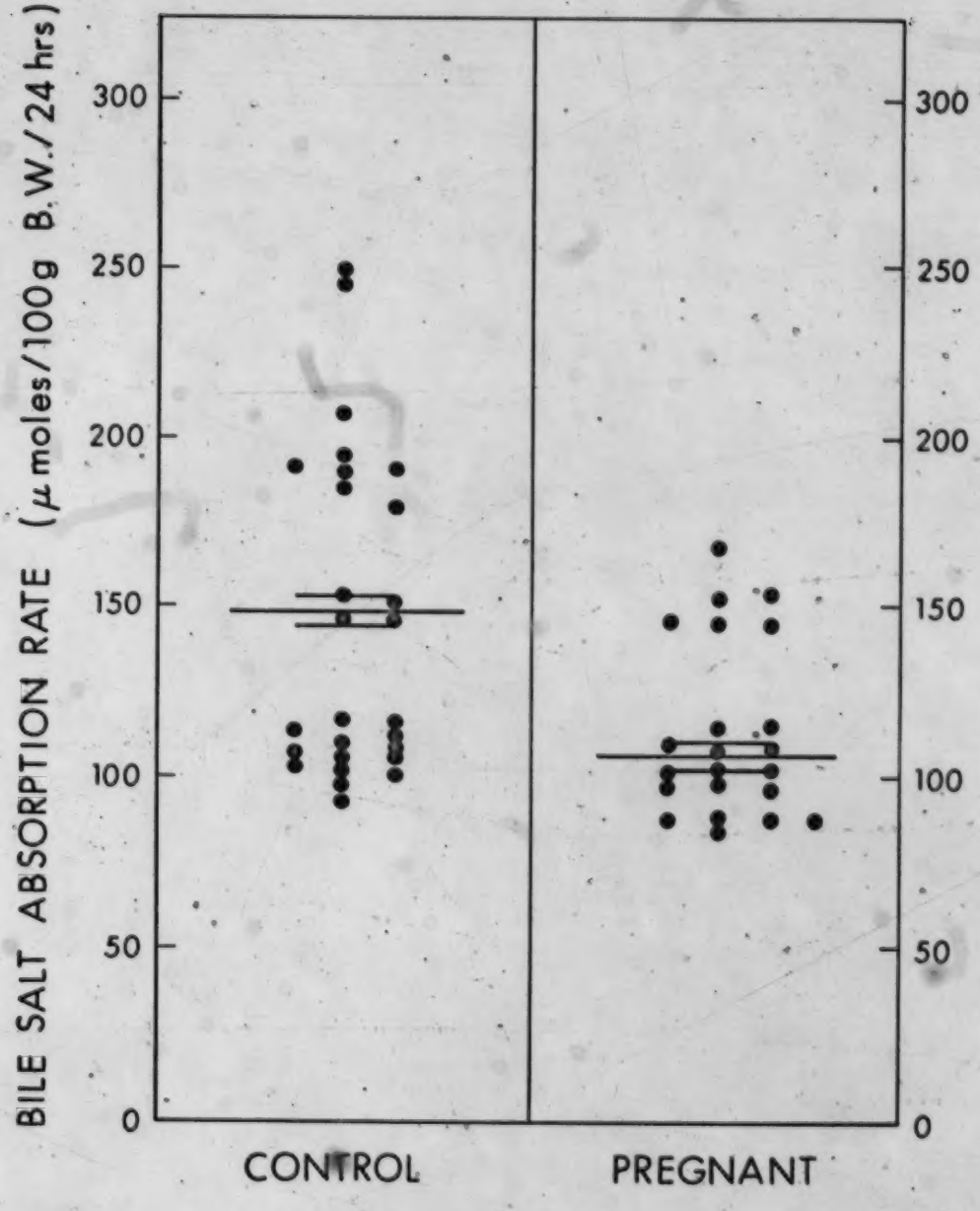


FIGURE 11

BILE SALT ABSORPTION RATES

(MEAN ± S.E.M.) IN CONTROL AND PREGNANT RATS

BILE SALT ABSORPTION

GROUP	ABSORPTION RATE µMoles/100g B.W.	ABSORPTION EFFICIENCY Percent
CONTROL		
No. of Rats	28	28
Mean	147.93	89.83
Std. Dev.	42.27	4.47
S.E.M.	7.99	0.84
PREGNANT		
No. of Rats	22	22
Mean	114.64	92.18
Std. Dev.	31.35	4.63
S.E.M.	6.68	0.99
t value	3.0264	1.7811
p value	< .01	N.S.*

TABLE VI

THE EFFECT OF PREGNANCY ON BILE SALT ABSORPTION

IN THE RAT

*N.S.: $p > .05$

Intestinal Absorption of Bile Acids

The absorption rate/24 hours in the pregnant group was 22% lower and this finding is statistically significant (Fig. 11 Table VI). However the Efficiency of Absorption is similar in both groups (Table VI) which indicates that the reduced absorption rate may be due to the unavailability of bile salts for absorption rather than inhibition of the bile acid active transport system in the ileum.

Basal Hepatic Bile Salt Synthesis

The results from measurements of basal hepatic bile salt synthesis per 24 hours are shown in Fig. 12 Table VII. In the pregnant group bile salt synthesis was significantly depressed by 36% and this finding would appear to be the only explanation for the reduction in pool size.

Bile Lipid Composition

The concentrations of the individual bile lipids in the first two hours bile sample are expressed as a molar ratio (moles %) of all three lipids. The Bile Acid content of the lipid fraction was significantly greater in the gravid than control animals but the phospholipid content was reduced by pregnancy. Cholesterol content was very low in all animals but was markedly reduced in the pregnant group. The character of these observed changes excludes dilution or concentration of whole bile as an explanation of these findings (Table VIII).

To determine if any overall change in cholesterol solubility occurs in pregnancy composition, data from both

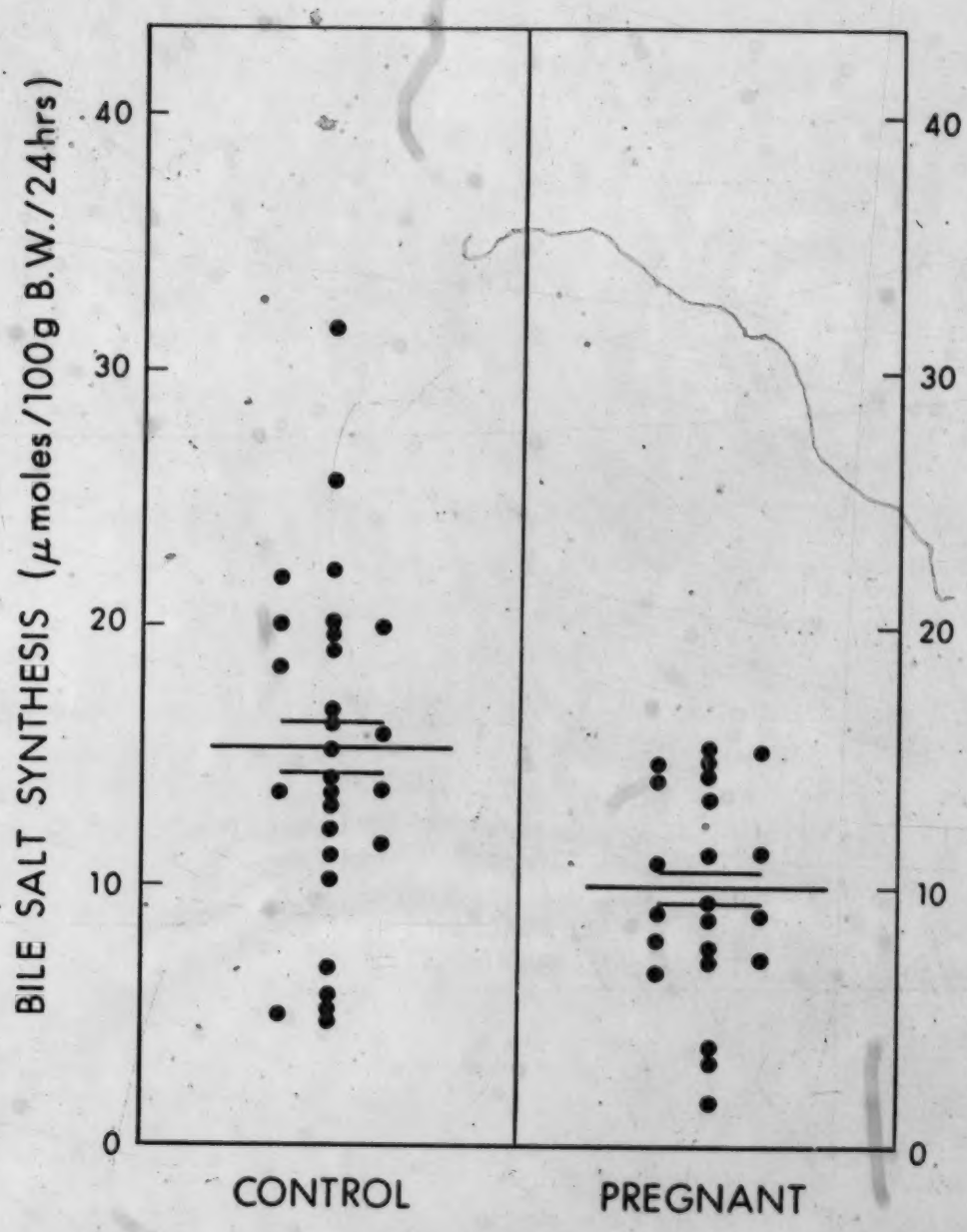


FIGURE 12

BILE SALT SYNTHETIC RATES

(MEAN ± S.E.M.) IN CONTROL AND PREGNANT RATS

BILE SALT SYNTHETIC RATES µMoles/100g B.W.

	CONTROL	PREGNANT
No. of Rats	28	22
Mean	15.35	9.90
Std. Dev.	6.27	4.07
S.E.M.	1.18	0.87
t value	-	3.4643
p value	-	<.01

TABLE VII

THE EFFECT OF PREGNANCY ON BILE SALT SYNTHESIS IN THE RAT

BILE LIPID COMPOSITION MOLAR RATIO %

GROUP	BILE SALT	PHOSPHOLIPIDS	CHOLESTEROL
CONTROL			
No. of Rats	26	26	26
Mean	88.25	10.48	1.26
Std. Dev.	2.32	2.17	0.31
S.E.M.	0.45	0.43	0.06
PREGNANT			
No. of Rats	22	22	22
Mean	90.45	8.91	0.87
Std. Dev.	2.92	3.02	0.36
S.E.M.	0.82	0.64	0.08
t value	-2.8490	2.0472	3.9290
p value	<.01	<.05	<.001

TABLE VIII

THE EFFECT OF PREGNANCY ON BILE LIPID COMPOSITION

IN THE RAT

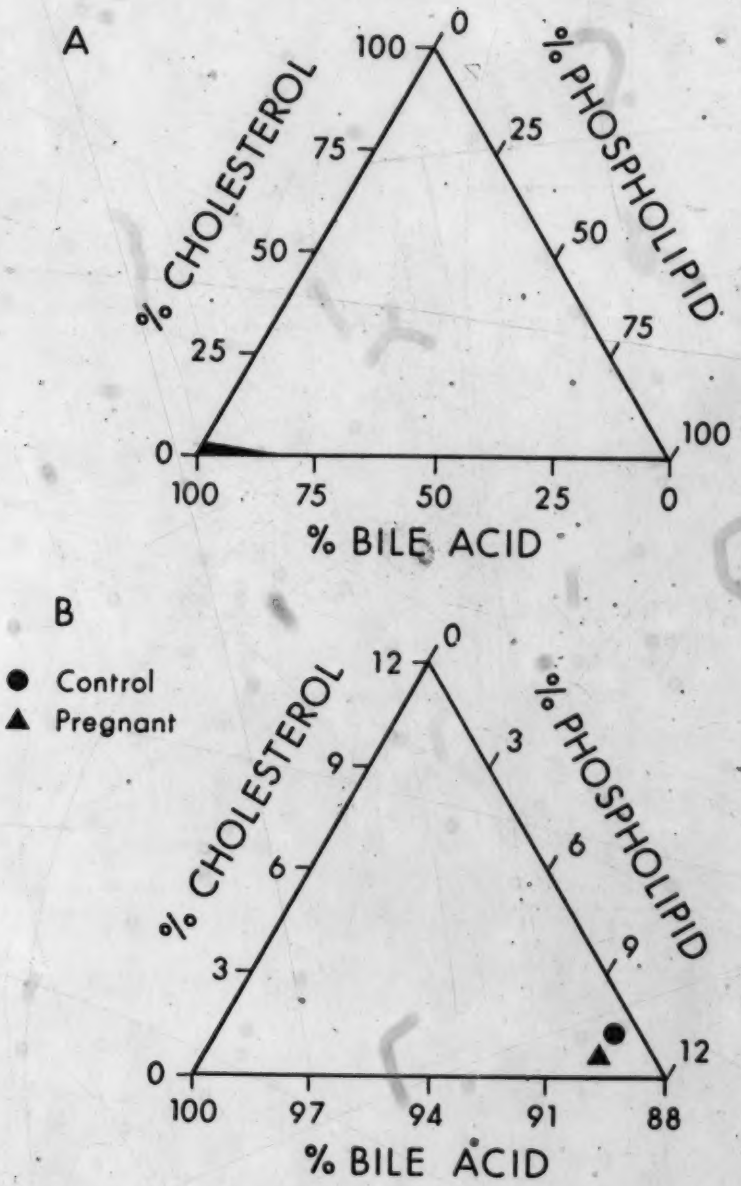


FIGURE 13

TRIANGULAR PHASE DIAGRAMS OF BILE LIPID COMPOSITION IN CONTROL AND PREGNANT RATS

groups were plotted on triangular coordinates as modified from Admirand and Small (20) (Fig. 13). In this species the three component system in bile is represented by the darkened area at lower left hand corner of the standard triangular coordinate graph. Therefore in order to compare solubility characteristics of the two groups composition data were plotted on a modified graph (Fig. 13). This reveals that in the rat cholesterol solubility at least was not adversely effected by the reduction of bile acid pool by pregnancy.

Summary

In the pregnant group relative to control there was a significant reduction in bile salt synthesis, pool size and secretion rate, but the enterchepatic cycle frequency was unchanged. The absorption rate of bile salts was also lower in the pregnant animals but the efficiency of absorption was similar to control group. Bile phospholipid and cholesterol secretion rates were significantly lower in the pregnant group and cholesterol solubility was not adversely affected. A general overview of the indices of the EHC of bile salts in control and pregnant rats is presented in Fig. 14.

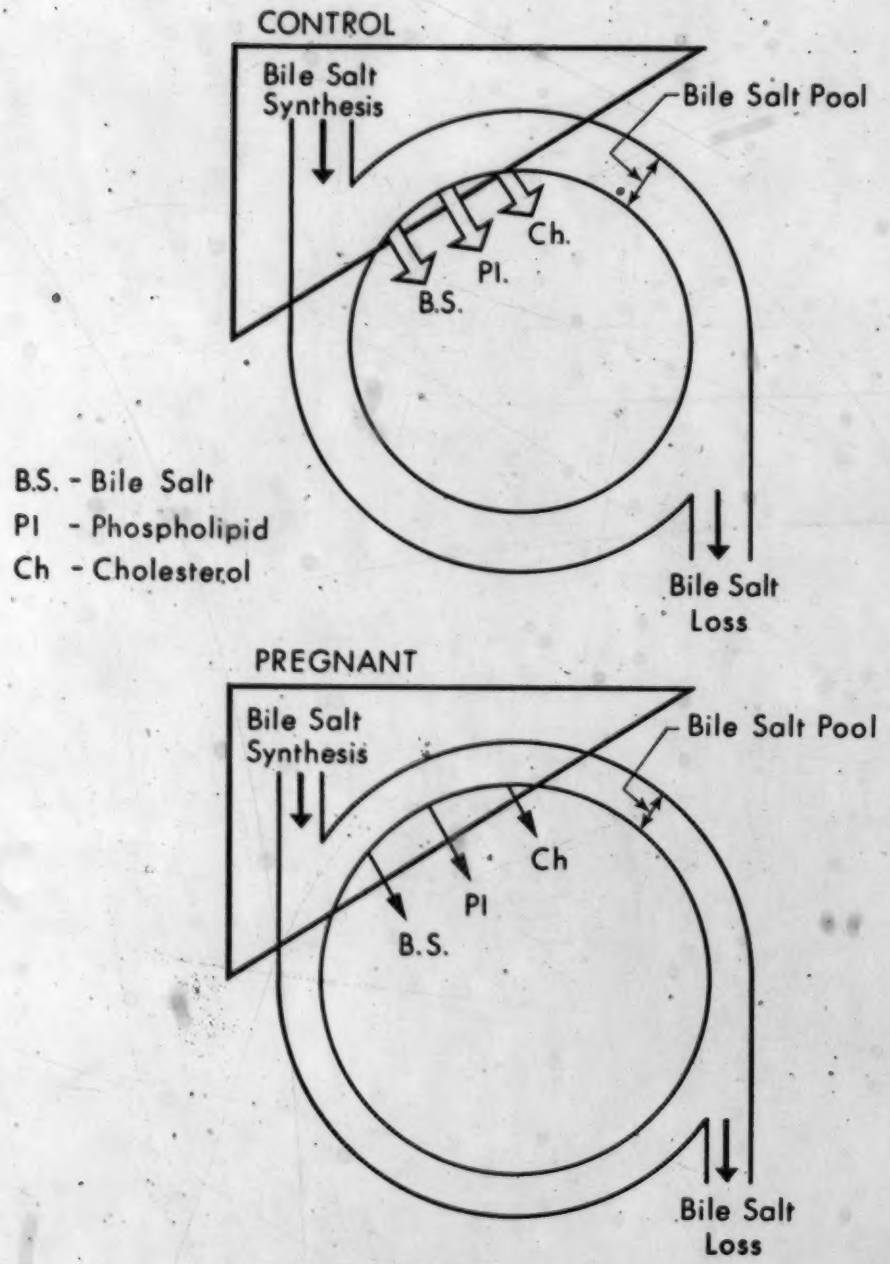


FIGURE 14

THE ENTEROHEPATIC CIRCULATION OF BILE SALTS IN CONTROL AND PREGNANT RATS

CHAPTER V

DISCUSSION

The methods used in measuring bile acid turnover in this study are similar to those used in an experiment on rats by Mok et al (52). A comparison of control data from both investigations reveals similar findings for bile acid measurements apart from the secretion rate which was lower in our study at 163.4 μmoles/100 gms BW/24 hrs. as compared to 185 μmoles and this difference may be a variable between colonies of rats. The 'washout method' used in the context of this study should have many advantages over the other methods available to study bile acid metabolism. While bile acid pool size and synthetic rate may be determined by Linstedt's isotope dilution technique (117) it may be an unsuitable method for a study such as this. In the isotope method serial bile sampling, during a steady state, is required in order to derive a specific activity decay curve. The short gestation period and the rapid fetal growth could vary conditions such that this system might be inapplicable in gravid rats. Furthermore bile acids are known to cross placental

barrier but whether or not they are concentrated by the fetus is unknown (118). This information is essential for an isotope study as transplacental migration and concentration of bile acids would introduce to the experiment further variables particularly in animals with variations in fetal numbers. An advantage of the direct method is that all studies are completed in a 24 hour period thus facilitating control and standardization of experimental conditions. While bile lipid secretion rates may also be measured indirectly by the duodenal perfusion method (50) this technique is elaborate and unsuitable for application to rats. Furthermore, direct measurement is considered more accurate.

In this investigation both groups of rats were maintained under similar conditions. The only single variable in the experiment was pregnancy hence any observed differences between the two groups must be considered due to parity or associated factors. It is therefore shown that pregnancy in the rat significantly reduced bile salt pool size, synthetic, secretion rates together with cholesterol and phospholipid outputs.

The significant reduction in bile acid biosynthesis has not been previously described in the gravid state though it has been suspected to occur from the similar finding after female sex hormone therapy (64). A number of separate mechanisms may be postulated for this diminution in synthetic rate for example:

(a) Impaired availability of cholesterol to the pool which

is utilized for bile acid synthesis.

(b) A partial deficiency or inhibition of the enzyme system producing bile acids.

(c) Diminished hepatic secretion of bile acids.

These mechanisms have not been studied during pregnancy and minimal data concerning the effects of female sex hormones is available from pharmacological experiments. A further difficulty in the explanation of this effect is that pregnancy and sex hormone therapy may produce suppression of bile salt synthesis by different mechanisms. Administration of sex hormones is known to suppress the endogenous sex hormone production from cholesterol (119) while in pregnancy increased steroidogenesis demands increased availability of cholesterol substrate. It is possible therefore that exogenous hormones and pregnancy have opposite effects on the size of cholesterol pool. Variation of cholesterol pool size under different conditions has been found to alter bile acid pool size and synthetic rate in rats and in humans (118). Whether or not significant changes in cholesterol pool size or biosynthesis occur during pregnancy is unknown but a lowering of pool size is suspected to accompany the gravid state not only due to the increased hormone synthesis but also estrogens may suppress cholesterol synthesis (120).

Cholesterol 7αhydroxylase is the rate limiting enzyme in the synthesis of bile acids from cholesterol. Reduced activity of this enzyme resulting in impaired catabolism of cholesterol is thought to explain the increase in serum and

tissue cholesterol levels found in some hypolipidaemic states (118). The serum cholesterol levels in humans (121) and rats (122) fluctuate widely throughout pregnancy and elevate towards its final stages but whether these effects result from changes in the cholesterol 7αhydroxylase activity in liver is unknown. Whilst the status of this enzyme is unknown during pregnancy a reduction in hepatic mass is excluded as a cause of the reduction in bile salt synthesis. Retention of bile acids within liver would also suppress this enzyme system but whether the hepatic bile salt level increases normally during pregnancy requires to be determined.

The reduction of bile acid pool size by pregnancy has not been previously described. Changes in size of bile salt pool have been found to occur with either an alteration of bile acid synthesis or reabsorption (52). The findings in this study indirectly exclude an increase in loss of bile acids and indicate that a low bile salt synthetic rate is the only causative agent in the reduction of the pool size. This reduction in the presence of a normal enterohepatic circulation cycle frequency may explain the reduced bile salt secretion rate. However the reduction in hepatic transport of bile acids by estrogen therapy (32,33,51) and bromosulphthalein excretion in pregnancy (87) suggests that suppression of the hepatocyte bile acid active transport system may also be contributing to their hyposecretion.

Lowering of biliary cholesterol and phospholipid secretion rates usually accompany a reduction of bile salt

secretion and this may explain their reduced outputs in the gravid rat. A sex difference of phospholipid concentration in human hepatic bile with a lower concentration in females has been reported (123) and is considered by Nilsson to indicate suppression of phospholipid synthesis by female sex hormones (42). The reduced secretion rate and biliary concentration of phospholipids associated with the diminished return of bile salts to the liver in the pregnant rat suggests that phospholipid synthesis may also be reduced. A reduction of cholesterol synthesis and pool size in pregnancy has been previously postulated in this discussion.

While the bile salt pool size was noticeably reduced by pregnancy cholesterol solubility in bile was not adversely affected as its secretion was also reduced. This finding is in marked contrast to that in humans where reduction in bile acid pool size by a similar magnitude would result in an increase in bile lithogenicity (53,54,55,56,57).

Interpretation of our data in relation to cholelithogenesis in humans requires a comparison of composition characteristics of rat and human bile. The bile acid characteristics are similar in both in that the dominant primary bile acids are cholic and chenodeoxycholic. In the rat they are conjugated almost exclusively with taurine as compared to the human where the glycine to taurine concentration ratio is between 1 and 6 (124). A further difference in bile composition is that rat bile is very dilute when compared with that of humans and solubility data are not available for model

ternary solutions having this degree of dilution. In addition to this very low levels of cholesterol are found in rat bile and this makes it difficult to relate changes in bile lipid composition to the area of cholesterol solubility and gallstone formation. A further difficulty in the use of the rat or similar animal model for the study of lithogenic factors in bile is that no animal species other than the baboon and humans are known to form gallstones spontaneously.

A final difficulty which arises, when relating changes in bile acid metabolism during pregnancy in rats to the clinical situation, is that the estrogen responses during pregnancy may not occur to the same degree or duration in rodents as in monkeys or humans (33).

Nevertheless, despite the considerable species differences this study demonstrates that real changes in bile acid turnover and bile composition occur in association with the gravid state. Should similar change in bile acid pool occur during human pregnancy supersaturation of bile with cholesterol would almost certainly be the result particularly in those women where biliary cholesterol levels normally approach saturation values.

CONCLUSIONS

1. Pregnancy caused a marked reduction in the bile salt synthetic rate in the rat.

2. The absorption rate of bile salts was lower in the pregnant group but this effect is due to the unavailability of bile acids for absorption rather than suppression of the ileal active transport system as the efficiency of absorption is unchanged.

3. As a result of this change in synthetic rate there was a significant reduction in bile salt pool size.

4. The EHC frequency of bile salts was unchanged by pregnancy.

5. Bile acid secretion was reduced by pregnancy probably as a result of the reduction in the bile salt pool.

6. Associated with the reduction in bile salt secretion was a reduction in outputs of bile phospholipids and bile cholesterol.

7. The finding of a reduced pool size of bile acids together with the low output of phospholipids suggests that phospholipid synthesis may also be reduced in pregnancy.

8. There was no adverse change in bile cholesterol solubility during pregnancy in rats due to its greater reduction in secretion rate.

9. Extrapolating from this experiment to the context of human gallstone formation it is postulated that if a similar reduction in bile acid pool size occurred during human pregnancy it would result in cholesterol supersaturation of bile particu-

larly in those patients where cholesterol levels normally approach saturation values.

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APPENDIX

BIOCHEMICAL ANALYSIS

Bile Acids

Total Bile Acid levels were determined enzymatically using Engert and Turner's modification (111) of Turnberg and Anthony-Moate's (112) 3αhydroxy steroid dehydrogenase technique for total bile acids.

Principle

The Bile Acids are 3αhydroxysteroids and react with 3αhydroxysteroid dehydrogenase in the presence of Nicotinamide adenine dinucleotide (NAD) to form Steroid Ketone + NADH. The reaction may be represented as

$$\text{Bile Acid} + \text{NAD} \underset{\text{3αhydroxy steroid dehydrogenase}}{\rightleftharpoons} \text{Steroid Ketone} + \text{NADH} + \text{H}^+$$

Completion of this reaction to the right is obtained using hydrazine as a ketone trapping agent. The NADH is estimated spectrophotometrically and its concentration is proportional to the quantity of bile salt present.

Reagents

1. Sodium pyrophosphate solution (0.1M). Adjust to pH 10.2 with 0.1 N. NaOH.
2. Hydrazine sulphate (1.0M). Add 3.4 ml of 95% hydrazine, 1.5 mls concentrated H_2SO_4, make volume to 100 mls with deionized water.
3. Nicotinamide Adenine Dinucleotide (6.8mM). Add 4.9 mgms to each 1 ml of deionized water.
4. Chenodeoxycholic Acid (CDCA 10.0mM. Sol.). Add 20.5 mgms to 4 ml deionized water, followed by 1 ml of 0.1 N. NaOH.
5. 3αHydroxysteroid dehydrogenase (Enzyme and Preparation) is a crude preparation of dried cells of Pseudomonas testosteroni. 50 mgms Alumina and 5 mls of 0.03M of tris-(hydroxymethyl) Aminomethane (Tris) and 0.01 M sodium Ethylene-diaminotetrocetate (E.D.T.A.). The resulting suspension was centrifuged for 20 minutes at 16,000 R.P.M. and the supernatant was used directly for the bile salt assay.

Procedure

For bile salt assay the reagents, standards and bile samples were added to test in the volumes (mls) outlined below.

	Blank	Test	Chenodeoxycholic Acid Standards		
Buffer	2.0	2.0	2.0	2.0	2.0
Hydrazine	1.0	1.0	1.0	1.0	1.0
NAD	0.5	0.5	0.5	0.5	0.5
Bile	0.0025	0.0025	-	-	-
Enzyme "Prep"	-	0.025	0.025	0.025	0.025
Boiled Enz. Prep	0.025	-	-	-	-
Chenodeoxycholic Acid (10.0mM)	-	-	0.01	0.03	0.05

The tubes were incubated for 60 minutes at 37°C in a water bath. The optical density was then read against the blank specimen in a Unicam S.P. 1800 Spectrophotometer at 340 Nm. A graph was constructed plotting CDCA standard concentration against absorbance and the quantity of bile salt in test sample was determined from the standard graph.

Phospholipids

The Phospholipid concentration was calculated from the total lipid phosphorus content of bile.

Principle

The phospholipid lecithin is extracted from bile with a mixture of chloroform and methanol and the extract is evaporated to dryness, the residue is digested with Sulphuric Acid and Hydrogen Peroxide. Phosphorus in the digested extract reacts with acid - Molybdate solution to form Phosphomolybdic acid. The Phosphomolybdic acid is reduced by Aminonaphthol-Sulfonic Acid to form a blue colour with an intensity proportional to the amount of phosphorous present.

Reagents

1. Chloroform/Methanol mixture Volume Ratio 2:1.
2. Sulphuric Acid 5 N. Add 145 mls of concentrated sulphuric acid to deionized water and make volume to 1 liter.
3. Dilute Sulphuric Acid. 1 ml concentrated sulphuric acid. Dilute to 2 liters with deionized water.
4. Hydrogen Peroxide 30%. Reagent grade.
5. Ammonium Molybdate Solution 2.5% W/V. Dissolve 12.5 gms ammonium molybdate in deionized water and make volume to 500 mls.
6. Sodium Bisulfite 15% W/V. Dissolve 30 gms reagent grade sodium bisulfite in deionized water and make volume to 200 mls.

7. Sodium Sulfite 20% W/V. Dissolve 20 gms anhydrous reagent grade sodium sulfite in deionized water and make volume to 100 mls.
8. Aminonaphthol-Sulfonic Acid Reagent. Add 0.5 gram 1,2,4 aminonaphthol-sulfonic acid to 195 mls of 15% sodium bisulfite and 5 mls of 20% sodium sulfite solution. Shake until dissolved.
9. Standard Phosphorus Solution 0.08 mgm/L. Dissolve 351.4 mgms of pure dry potassium dihydrogen phosphate in 20 mls of $5N.H_2SO_4$. Make volume to 1 liter with deionized water.

Procedure

1. The volume of bile (1 ml) was added drop by drop to 22 volumes of chloroform/methanol mixture while mixing in a vortex. The mixture was then allowed to stand at room temperature without agitation for 5 minutes. The tube was then stoppered and shaken for 30 seconds, brought to 25 volumes by addition of choloroform/methanol -- and the mixture was then allowed to stand for 5 minutes after which the extraction was complete. 5 ml of dilute sulphuric acid was added to the mixture and the tube was inverted 10 times without shaking. The mixture was allowed to stand for 10 minutes for equilibration of the phases, followed by centrifugation at 2,000 R.P.M. for 15 minutes. The lower chloroform phase contains all the lipids and 5 mls of this phase were transferred to a digestion apparatus and the solution evaporated to dryness: 2.5 mls of $5N.H_2SO_4$ were then added to the residue and the mixture was subjected

to slow boiling. When the colour of mixture turned brown or black and remained constant the tube was removed from digestion apparatus, allowed to cool slightly, and 1 drop of 30% hydrogen peroxide was added to the digestion mixture. The tube was then replaced on the digestion apparatus and the heating continued. Further hydrogen peroxide was added when necessary until the mixture became colourless. The digestion was continued for a further 10 minutes after the last addition of hydrogen peroxide.

2. The standard was prepared by transferring 0.5 mls of the phosphorus standard solution to a digestion tube and adding 2.5 mls of 5N.H_2SO_4. 2.5 mls of Conc Sulphuric acid served as blank. The same number of drops of hydrogen peroxide were added to the standard and blank tubes as were required for the phospholipid test sample. The tubes were then placed on the digestion apparatus and the contents boiled for ten minutes. The contents of each digestion tube were then diluted with a few mls of deionized water cooled to room termperature, and then transferred to 25 ml volumetric flasks with repeated washing so that the flask was approximately half filled. 2.5 mls of Ammonium molybdate solution were added to each volumetric flask followed by 1 ml of Aminonaphthol Sulfonic acid reagent. The contents of each flask were then diluted to the 25 ml mark and allowed to stand for 5 minutes. The optical density of each specimen was then measured with a Unicam SP. 1800 Spectrophotometer at a wavelength of 675nm.

Calculation of Total Lipid Phosphorus and Phospholipid Content

The standard contained 0.04 mgms phosphorus.

$$\text{Lipid P. mgm/100 mls bile} = \frac{\text{OD (unknown)}}{\text{OD (standard)}} \times 0.04 \times \frac{18}{5} \times 100$$

$$\text{Phospholipid mgms/100 mls bile} = \frac{\text{OD (unknown)}}{\text{OD (standard)}} \times 14.4 \times 25$$

The molar concentration of phospholipids in sample was then determined (1 mole lecithin--793 mgms).

Cholesterol

Though cholesterol exists in bile only in the free form the Abell method (115) of estimating serum cholesterol was applied as this method is used routinely on a daily basis in the laboratory.

Principle

In this method the serum or bile is treated with alcoholic potassium hydroxide to liberate cholesterol from lipoprotein complexes and to saponify cholesterol esters. Cholesterol is then extracted into a measured volume of petroleum ether. An aliquot of the petroleum ether extract is measured utilising the Liebermann-Burchard reaction, and the green color is read spectrophotometrically.

Reagents

1. 95% Ethyl alcohol, redistilled.
2. Petroleum Ether (P.Pt. 68°C). Reagent Grade.
3. Glacial Acetic Acid - Reagent grade.
4. Concentrated Sulphuric Acid - Reagent grade.
5. Acetic Anhydride - Reagent grade free from HCL.
6. Potassium Hydroxide Solution, 33% W/W. Dissolve 10 grams of reagent grade KOH in 20 ml of deionized water.
7. Alcoholic Potasium Hydroxide (KOH Solution). Add 6 ml of 33% KOH solution to 94 ml of 95% ethyl alcohol (Prepare immediately before use).
8. Standard Cholesterol Solution (0.4 mgms/ml). Dissolve 100

grams of cholesterol (Recrystallized 4 times from absolute methanol and dried to a constant weight) in absolute methanol. Make volume to 250 mls with methanol.

9. Modified Liebermann-Burchard Reagent. Chill 20 volumes of acetic anhydride below 10°C in a glass stoppered flask. While shaking flask slowly add 1 volume to concentrated sulphuric acid and keep the mixture cool for 9 minutes. Then add glacial acetic acid (10 volumes), allow to warm to room temperature. Use reagent within one hour of preparation.

Procedure

0.5 ml of bile is measured into a stoppered centrifuge tube to which is added 5 mls of alcoholic KOH. The tube is well shaken and incubated in a water bath at 37-40°C for 55 minutes. After cooling to room temperature 10 mls of petroleum ether are added and well mixed. 5 mls of water are then added and the tube is shaken for 1 minute. The tubes are then centrifuged at 2,000 R.P.M. until the emulsion breaks and two clear layers appear (usually about 10 minutes). Duplicate 4 ml aliquots of the petroleum ether layer are then transferred to large dry test tubes. The petroleum ether is then evaporated under a nitrogen stream in a water bath at 60°C.

Standards are prepared and run through the procedure along with the test samples as follows. Duplicate 5 ml samples of standard Cholesterol solution (0.4 mgms/ml) and 0.3 mls of 33% KOH solution are added to 25 ml glass stoppered centrifuge

tubes. The contents are well mixed and incubated for 55 minutes at 37-40°C. 10 ml of petroleum ether and 5 ml of deionized water are added and the tubes shaken vigorously for one minute. After centrifugation 1,2 and 3 ml samples of the petroleum ether layer are measured into dry test tubes and evaporated to dryness providing standards equivalent to 0.2, 0.4, and 0.6 mgms cholesterol. After cooling to room temperature 6 mls of modified Liebermann-Burchard Reagent is added at one minute intervals to the standard and sample tubes containing the dry residues and also to a blank test tube. The tubes are then stoppered and mixed using a shaker in a dark corner of laboratory. Exactly 30 minutes after adding the reagent the optical density of each sample is read against the blank in a Unicam 1800 Spectrophotometer at a wavelength of 620nm.

Calculation of Results

The optical density equivalent to 1 mgm of cholesterol (S) is calculated from the readings of the standards.

$$\frac{\text{OD of Standard}}{\text{Mgm Cholesterol in Standard}} = S.$$

The S. Value for all standards must agree within 4%, and the average of all values is used for calculating the Cholesterol content of sample as follows:

$$\frac{\text{OD of unknown (Bile)}}{\text{X.}} \times \frac{10}{\text{Vol. Petrol Ether Aliquot}} \times \frac{100}{\text{Vol. Bile Sample}}$$

= Mgm Cholesterol/100 mls of bile

The molar concentration of cholestrol in sample is then determined (1 mM cholesterol = 387 mgms).

STATISTICAL METHODS

The arithmetic mean, standard deviation and standard error of mean was calculated for each series of observations as follows:

Arithmetic Mean

$$\bar{x} = \frac{\Sigma X}{N}$$

$\bar{x}$ = Mean

Σ = Sum of

X = Observation

N = Number of observations

Standard Deviation

$$S = \sqrt{\frac{\bar{x}^2 - \frac{(X)^2}{N}}{N-1}}$$

S = Standard Deviation

X = Observation

$\bar{x}$ = Mean

Standard Error of Mean

$S\bar{x}$ = Standard Error of Mean $\quad S\bar{x} = \frac{S}{\sqrt{N}}$

S = Standard Deviation

N = Number of observations

The unpaired Students 't' test was used to determine whether the difference between the two means was significant. The Test is:

$$t = \frac{\text{Difference between both Means}}{\text{Standard Error of Mean Difference}}$$

$$t = \frac{\overline{x1} - \overline{x2}}{S_{\overline{d}}}$$

$\overline{x}_1$ = Mean of sample 1.

$\overline{x}_2$ = Mean of sample 2.

$$S_{\overline{d}} = \frac{S_1^2 + S_2^2}{N_1 + N_2}$$

$S_{\overline{d}}$ = Standard Error of Mean Difference

$$S_1 = \sqrt{\frac{\Sigma X_1^2 - \frac{(\Sigma X_1)^2}{N_1}}{N - 1}}$$

$$S_2 = \sqrt{\frac{\Sigma X_2^2 - \frac{(\Sigma X_2)^2}{N_2}}{N - 1}}$$

Having calculated the 't' value -- the probability value was obtained from the 't' table for the appropriate degrees of freedom (DF)

In this context DF = $(N_1 + N_2) - 2$

(DF = The number of independent random selections that may be made from a sample.)

SIGNIFICANCE OF PROBABILITY LEVELS

Probability (p) of differences being due to chance.		Interpretation
p = more than 5%	$p > (0.05)$	Significant difference not proven.
p = 5% to 1%	$p \leq (0.05)$	Difference is just significant.
p = 1% to 0.1%	$p \leq (0.01)$	Difference is significant.
p = 0.1% or less	$p < (0.001)$	Difference is highly significant.

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